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Physical Forces from the Extracellular Matrix Influence Breast Cancer Cell Response to Doxorubicin

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Abstract

Physical Forces from the Extracellular Matrix Influence Breast Cancer Cell Response to Doxorubicin

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Cancer is a complex disease capable of affecting multiple organs and is driven by numerous factors. Certain 'hallmarks of cancer' have been identified which describe biological conditions that lead to tumor development and characteristics that often follow tumorigenesis. These hallmarks have been revisited to describe the role that the extracellular matrix (ECM) plays in each. Such observations make it evident that the ECM is an important factor in tumor initiation, progression, and metastasis and can no longer be ignored in the search for a cure. Studies aimed at characterizing the role physical cues play in tumor development have considered ligand variety, ligand density, substrate composition, and substrate stiffness. These studies frequently utilize hydrogels as a culture platform given the biological relevance and diversity achievable through such a platform. Though the stiffness of hydrogels can be attenuated at the onset of an experiment, few systems are able to alter stiffness once gelation is complete. This makes any study of progressive changes in ECM stiffness difficult and largely restricts the study of certain temporal aspects of tumor progression *in vitro*. Recently, a Matrigel-alginate hydrogel system has been described whereby progressive modulation of hydrogel stiffness can be

achieved using liposomes loaded with gold nanorods and near infrared light. In this study we utilize this hydrogel system to thoroughly investigate the role that ECM stiffness has on breast cancer response to doxorubicin. We sought to observe how progressive stiffening of the ECM affected breast cancer cell response to clinically relevant chemotherapeutics in a system that allows for minimal perturbation of cells during the stiffening process. Our results showed that breast cancer cells exhibiting a mesenchymal phenotype had a stiffness-dependent resistance to the chemotherapeutic doxorubicin. Mathematical modeling was used to determine reduced growth rate alone was not sufficient to explain this stiffness-dependent resistance, suggesting an additional mechanism associated with the mesenchymal phenotype is responsible.

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Chapter 1: Introduction[1]

1.1 The Extracellular Matrix

The extracellular matrix (ECM) encompasses all the non-cellular components of a tissue that support and give structure to the cells[1]. It is a vital component in all animals that functions as a scaffold for cells to adhere to and a medium for transmitting biochemical and biomechanical cues. The ECM is separated into two domains: the interstitial connective tissue matrix[2] and the basement membrane[3]. Interstitial connective tissue matrix fills the interstitial space between cells and is composed primarily of collagens I, III, V, VI, VII, XII[2]; fibronectins[4-6]; and proteoglycans, mostly chondroitin sulfate[7,8] and heparin sulfate[9,10]. The basement membrane is a specialized ECM that separates epithelium from mesenchyme, guides cellular differentiation[11], stores growth factors[12], and plays a role in cell proliferation and migration. It is vital for epithelial cells to properly orient themselves. Epithelial cells will anchor their basal side to the basement membrane with the apical side being exposed to a fluid-filled lumen. The basement membrane is primarily composed of type IV collagen, laminin, nidogen/entactin, and perlecan. Collagen[13] and laminin[14] are the major structural components, as they are able to independently self-assemble into networks. Nidogen/entactin and perlecan further stabilize the basement membrane by binding to both collagen and laminin, thus bridging the two networks and increasing structural integrity[15-18].

The early discoveries of the ECM can be attributed to mostly to gross characterization and light microscopy. Nageotte and collaborators were able to isolate what we now know to be collagen from rat tissue in the 1930's[19]. This substance was brought to their attention when they noted its ability to reversibly solubilize and reconstitute into fibrils. During this time, the invention of the electron microscope[20] and advances in x-ray diffraction[21] would make it possible for researchers to more fully understand the structure of ECM components and how they might interact with one

[1] Portions of this chapter were adapted from M. H. Joyce, S. Allen, L. Suggs and A. Brock, "Novel Nanomaterials Enable Biomimetic Models of the Tumor Microenvironment," *Journal of Nanotechnology*, vol. 2017, pp. 1-8, 2017. M. H. Joyce, S. Allen, L. Suggs and A. Brock wrote this review article.

another *in vivo*. Richard Bear[22] studied collagen fibrils using x-ray diffraction and found they had a 64 nm repeating period, and this finding was confirmed by Hall et al.[23] via electron microscopy. Schmitt et al.[24] utilized both x-ray diffraction and electron microscopy, as well as chromatography to show that collagen molecules could transition from fibrils into bundles they referred to as segment long spacings (SLS), and vice-versa. The same year, multiple groups used x-ray diffraction patterns of collagen fibrils to refine this model to the triple-chain collagen helix we know today[25-27]. Laminin wasn't discovered until 1979, and its characterization followed a much different path from collagen given the significant advances in technology. Laminin was first isolated from the Engelbreth-Holm-Swarm (EHS) sarcoma, a mouse tumor that was shown to produce ECM basement membrane[28,29]. It was identified when researchers found that neutral buffers extracted very little type IV collagen from these tumors, but a substantial amount of non-collagenous proteins. Ion exchange chromatography was used to determine the amino acid composition, polyacrylamide gel electrophoresis was used to determine approximate molecular weight, and immunofluorescence was used to localize laminin to the basement membrane[29].

The exact composition of the ECM depends on a number of factors that also influence resident cell phenotype such as mechanical forces, biochemical signals, oxygen requirements, and pH. Such factors vary across species and even across tissue types within species, so the ECM composition is highly tailored to the tissue type that it is found in. Tailoring the ECM is one control mechanism the tissue as a whole has on regulating local cell phenotype, migration, and proliferation[30-33]. Collagen is the major insoluble fibrous protein in the ECM[34], and the main structural element of the ECM[1], however there are numerous types that help shape the physical characteristics of a tissue type. Types I and III collagens have tremendous tensile strength, which makes them the ideal components for the ECM of tendons that connect muscle to bone[34]. On the other hand, type II collagen fibrils are smaller in diameter and orient randomly, giving the ECM of cartilage tissues their compressibility and resistance to deformations in shape[34]. These differences in ECM composition extend beyond variation in collagen types; the proteoglycan profile of skeletal muscle tissue, for example, is dominated by the small leucine-rich proteoglycan

family[35], whereas chondroitin sulfate proteoglycan constitutes the major population of proteoglycans in the central nervous system[36]. Glycosaminoglycans (GAGs) are the primary polysaccharide found in vertebrate ECM, however chitin (poly-N-acetylglucosamine) and cellulose are the main polysaccharides found in insect and plant ECM, respectively[37].

ECM composition contributes significantly to the overall physical properties of the tissue, including its stiffness, viscoelasticity, and pore size. **Table 1.1** shows stiffness measurements of various tissue types and illustrates the contribution of ECM to the stiffness of each tissue. The measurements listed in **Table 1.1** were collected primarily using atomic force microscopy (AFM) and magnetic resonance elastography (MRE)[38]. Atomic force microscopy is a form of scanning probe microscopy[39] that skims along the surface of a sample and, by tracking the deflection of laser light off the probe tip, can apply indentations along a samples and calculate the force required to do so and approximately how stiff the material must be to resist that force[40]. Magnetic resonance elastography works by inducing mechanical shear waves into the tissue, imaging with phase-contrast MRI techniques, and processing the wave images to calculate a measurement of stiffness[41]. Tissue viscoelasticity is dependent on the structural proteins of the ECM, crosslinking[42,43], folding/unfolding of ECM proteins[44,45], and flow of fluid through the ECM[43]. This property is especially important in softer materials and tissues, as compliant samples can lead to inaccurate measurements of stiffness when using techniques like nanoindentation[46]. Measurements of the viscoelasticity of ECM or ECM substitutes is typically performed using a rheometer to measure shear stresses and strains that are applied to the sample[47]. The porosity of the ECM helps dictate what size molecules and cells can move through the ECM. Since the pore size can be as small as a few nanometers, electron microscopy is a method commonly used to measure the average pore size and porosity of ECM.

1.2 EXTRACELLULAR MATRIX PLAYS CRUCIAL ROLES IN PHYSIOLOGY, DEVELOPMENT, AND DISEASE

During embryogenesis and early development, cells rely on physical cues, chemical gradients, and electrical signals for differentiation and morphogenesis. The ECM provides physical cues and facilitates chemical signaling during normal tissue development[48]. This is probably most evident in cardiomyocytes and myoepithelial cells lining blood vessels, which are highly sensitive to stretch and tension. Myocardial cells have been shown to increase their size and protein production upon exposure to cyclical stress, as opposed to myocardial cells that are grown in a static environment[49]. Cyclical stretching of cardiomyocytes induces phosphorylation of pathways involved in differentiation and proliferation such as ERK, JNK, FAK, and p38 MAPK[50]. Chondrocytes also require mechanical tension for proper development and maturation. Wong et al.[51] demonstrated that cyclical tension activates the Cbfa1/MMP-13 pathway and increases expression of terminal differentiation hypertrophic markers such as COMP and lubricin. Even neurons and glial cells respond to the mechanical properties of the tissue microenvironment to maintain normal physiological function[52-57]. Landmark studies in the field of neuronal development have shown how tension along axons and other mechanical cues contribute to organization of the nervous system[58-60].

Dysregulation of structure, function, or composition of the ECM contributes to many various diseases and conditions[61]. The mechanical properties of tissues vary widely among different physiological and pathological states[62,63]. Fibronectin has been shown to play a role in cardiovascular disease and tumor metastasis[64,65]. In the liver, hepatic fibrosis will cause stiffening of the ECM[66,67]. Similarly, patients with chronic obstructive pulmonary disease (COPD) have been reported to have decreased elastin[68-70] and proteoglycans[71] with increased collagen content in the alveoli, leading to a stiffening of the lung tissue and decreased elasticity. A detailed analysis of the ECM composition of COPD patients further showed decreased expression of type I collagen in the large and small airways, lower versican fractional area in distal parenchyma regions, and higher fractional areas of fibronectin and tenascin in large and small airways[72].

Tumors are typically stiffer than their surrounding healthy tissue and this is due, in part, to increased ECM deposition, ECM remodeling, and increased contractility of cells in the tumor microenvironment[73-75]. Tumor cells also secrete matrix metalloproteases (MMPs)[76,77] that break down surrounding basement membrane, releasing sequestered growth factors such as vascular endothelial growth factor (VEGF)[77,78] from the matrix. VEGF promotes the branching growth of blood vessels through angiogenesis, which further increases interstitial tissue pressure through added cell density. This leads to a positive feedback loop where tumor-associated stiffening causes the release of growth factors, which leads to increased interstitial pressure from proliferation, angiogenesis, and cellular contractility[73,79-81]. Similar changes have been observed in ECM of mammary tissue progressing through the stages of breast cancer[82-88]. Healthy mammary gland tissue is characterized as have a basement membrane composed of laminins, type IV collagen, nidogens, and perlecan with an interstitial ECM composed of fibrillar collagens (types I, III, and V), fibronectin, decorin, and biglycan. During cancer progression, MMPs and other ECM remodeling enzymes breakdown the basement membrane[89] leading to decreased levels of type IV collagen[83,84] and laminin-111[90]. The surrounding interstitial ECM gradually stiffens due to increased deposition of fibrillar collagen (types I, III, and V) and fibronectin along with elevated LOX activity[74]. Matricellular proteins that promote tumor cell survival including tenascin C, periostin, osteopontin, SPARC, and thrombospondin-1 are upregulated[91], as are the versican[92], syndecan-1[93], and glypican-1 proteoglycans[94]. These changes in mammary tissue ECM composition promote tumor growth and progression, which leads to a positive feedback loop that leads to further tissue stiffening and disease progression.

The difference in stiffness between healthy tissue and tumors may be significant and may be detectable by physical palpation. In fact, palpation remains a time-tested diagnostic tool for physicians as a means of quickly scanning the body for differences in the mechanical properties that would signify healthy or abnormal tissue. It is still used as a standard medical test to detect breast tumors[95] and liver tumors[96].

1.3 CELLS SHARE A RECIPROCAL SIGNALING RELATIONSHIP WITH THEIR EXTRACELLULAR MATRIX

The ECM exists in a state of dynamic equilibrium, as cells are constantly breaking down components of the ECM and secreting new ECM proteins[31]. Fibroblasts secrete and organize type I and III collagens, elastin, fibronectin, tenascin, hyaluronic acid, decorin, and additional proteoglycans to ensure structural integrity of interstitial ECM[1]. Conversely, cells also produce MMPs to break down ECM to facilitate cell migration and the release of growth factors that are sequestered in the ECM. Cells and their surrounding ECM are tailor made for one another. For example, smooth muscle cells assume an elongated cell shape based on interactions with the $\alpha_v\beta_3$ integrin receptor and tenascin-C, which promotes EGF-dependent survival[97]. Roskelley et al.[98] further describe a dynamic signaling hierarchy that occurs during mammary gland development. First they describe how a mammary cell must detach from its rigid substrate to allow for changes in cytoskeletal and nuclear architecture. This leads to integrin-dependent biochemical signals (FAK phosphorylation, MAP kinase activation, and transient increase in AP1 transcription factor activity) which activate ECM-responsive elements such as BCE-1. Signals initiating in the cell nucleus are conveyed to the surface membrane where changes to the ECM are executed; this might involve increasing MMP production to breakdown ECM or increasing secretion of structural ECM proteins. Tumor cells have also been shown to take advantage of this reciprocal relationship between cells and ECM. Fullár et al.[99] found that cancer-associated fibroblasts (CAFs) produced significant amounts of laminin-1 in response to signals sent from cervical cancer cells (CSCC7) that predominantly migrated towards and produced integrin receptors ($\alpha6\beta4$) for laminin.

1.4 RESISTANCE TO CHEMOTHERAPEUTIC TREATMENTS

Breast cancer is the second leading cause of cancer death among women[100]. It is estimated that breast cancer will have seen 268,670 new cases emerge and caused 41,400 deaths in 2018 in the U.S. alone[100]. One of the most challenging aspects of treating cancer is the fact that small populations of cancer cells may be resistant treatment and persist after treatment has finished. These cells survive the initial round of treatments, continue to proliferate, repopulate the tumor

(disease relapse), and may metastasize to other regions. Only 10% of malignant tumor types can be eradicated by chemotherapy, leaving the other 90% with varying degrees of improved survivability[101]. Current treatment regimens will usually include administering a cocktail of chemotherapeutics to the patient prior to (neoadjuvant) or immediately following (adjuvant) surgical resection of the tumor in an attempt to fully eradicate the disease. However, nearly 50% of all cancer patients are afflicted with a malignancy that is intrinsically resistant to chemotherapeutic treatment[101]. Most of the remaining half will stop responding to treatment as tumor cells acquire resistance to the chemotherapeutics being used. Intrinsically resistant tumor cells have pre-existing mutations that confer a survival advantage during initial treatment, whereas cells with acquired resistance (also known as therapy-induced resistance) undergo changes in response to treatment that ultimately decrease the effectiveness of the drug. A good example of intrinsic resistance is seen in tumor cells that carry the BRAF V600E mutation, which renders those tumor cells resistant to BRAF inhibitors[102,103]. Studies that selected for resistant populations through progressively increased dosing with topoisomerase II poisons (daunorubicin, doxorubicin, melphalan) showed that the surviving cells overexpressed the multi-drug resistance 1 (MDR1) gene which increased cell resistance through P-170-mediated MDR[104-106]. The molecular mechanisms behind cancer cell resistance to chemotherapeutic treatment include transporter pumps, activation of oncogenes, suppression of tumor suppressor genes, mitochondrial alteration, DNA repair, autophagy, epithelial-mesenchymal transition (EMT), cancer stemness, and exosomes[107-109]. In the present study, we focus primarily on the chemotherapeutic drug, doxorubicin (trade name Adriamycin®). This anthracycline functions by disrupting topoisomerase-II-mediated DNA repair[110,111], generating free radicals to damage cellular membranes[112,113], and inducing histone eviction[114]. It has proved useful as a broad-spectrum chemotherapeutic in neoadjuvant breast tumor therapies, but it is known to induce resistant cells that can cause disease relapse. Mechanisms of doxorubicin resistance may involve ABCB1 (MDR1, Pgp)[115], ABCC1 (MRP1)[116-118], ABCC2[118], ABCC3[118], ABCG2, and RALBP1[119] transporters, as well as amplification of TOP2A[120-122]. Another chemotherapeutic agent used in

this study is paclitaxel (trade name Taxol®), a member of the taxane class. This is a microtubule stabilizer that kills cancer cells by polymerizing and stabilizing mitotic spindles in dividing cells, thus preventing them from clearing the mitotic checkpoint[123]. Resistance to paclitaxel has been shown to arise through a variety of mechanisms. The mechanisms that are most well-studied include alterations to expression of IL-6 and IL-8 cytokines, alterations in β-tubulin, alterations in genes involved in apoptosis (BCL-2, BCL-XL, BAX, BAD, and p53), and overexpression of P-glycoprotein[124]. Many other mechanisms of paclitaxel resistance have been proposed as well including changes in fibroblastic growth factors[125], increased expression of transmembrane receptors[126,127], increased expression of adhesion molecules[128], increased expression of certain cell signaling molecules[128-134], increased expression of nuclear proteins[134-136], and the switch from apoptotic to autophagic cell death[137]. This list is by no means exhaustive, but illustrates the complex nature of treating cancer patients with modern chemotherapeutics.

1.5 THE EXTRACELLULAR MATRIX INFLUENCES HOW TUMOR CELLS WILL RESPOND TO CHEMOTHERAPEUTIC TREATMENT

Cancer is a complex disease capable of affecting multiple organs and is driven by numerous factors. Hanahan and Weinberg[138,139] have summarized and defined certain 'hallmarks of cancer' which describe biological conditions that lead to tumor development and characteristics that often follow tumorigenesis. Pickup et al.[140] revisited these hallmarks and described the role that the extracellular matrix (ECM) plays in each. Such observations make it evident that the ECM is an important factor in tumor initiation, progression, and metastasis and can no longer be ignored in the search for a cure. Studies aimed at characterizing the role physical cues play in tumor development have considered ligand variety, ligand density, substrate composition, and substrate stiffness[141-147]. More recent studies are focusing on understanding how the physical cues from the microenvironment affect how cancer cells will respond to chemotherapeutic treatment. Shin & Mooney[144] investigated how ECM stiffness would affect chemosensitivity of myeloid leukemia cells lines by culturing them within alginate hydrogels that not only varied in stiffness, but in

functionalizing peptide. What they were able to show was that the myeloid leukemia cell lines challenged with varying chemotherapeutics would fall into one of three categories: ligand sensitive, ligand and matrix stiffness sensitive, or mechanics independent. Zustiak et al.[148] cultured multiple cancer cell lines on top of collagen-coated polyacrylamide hydrogels that ranged from 1 – 100 kPa in stiffness. These samples were treated with paclitaxel to observe how ECM stiffness would modulate drug sensitivity. Their results showed only found HeLa and SY5Y cell lines to have a stiffness-dependent resistance to paclitaxel out of the 10 cell lines tested, however their study was limited in that the cells were cultured on top of the hydrogels and not embedded within them. Rice et al.[149] performed a very similar study with pancreatic ductal adenocarcinoma (PDAC) cell lines cultured on 1, 4, and 25 kPa polyacrylamide hydrogels. In their study, they found that the PDAC cell lines they used had a stiffness-dependent increase in resistance to paclitaxel. To uncover the mechanism behind the observed stiffness-dependent resistance, they measured total expression of vimentin and nuclear localization of YAP/TAZ. Their results showed that the PDAC cells they cultured on stiffer hydrogels increased expression of vimentin and had higher levels of nuclear localized YAP/TAZ.

YAP (yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) are functionally similar transcriptional co-activators capable of inducing EMT[150]. As the Hippo pathway effector, YAP has been shown to promote metastasis through its TEAD-interaction domain[151] and upregulation of EGF receptor[152] (**Fig 1.1**). LATS1/2 kinases are members of the Hippo pathway that will phosphorylate, and thus inhibit, YAP[153,154]. When YAP is phosphorylated, it will remain in the cytoplasm of the cell where it will eventually be marked for proteasome degradation. Once translocated to the nucleus, however, it can perform its role as a transcriptional co-activator that regulates organ size, cell proliferation, and EMT. YAP has been shown to drive these effects through targeting of ABCB1, ANKRD, CAT, GPATCH4, LMNB2, PTGS2, TXN, WSB2, and more specifically AXL, CDKN2C, CTGF, CYR61, DAB2, DLC1, TSTL1, LHFP, SDPR, SERPINE1, SLIT2, TGFB2, and THBS1 in breast tumor samples[155,156].

Tissue	Elastic Modulus (E, Pa)	References
Brain, white matter	(~2.25) x 10^2	Christ et al.[155]
Brain, gray matter	(~3.4) x 10^2	Christ et al.[155]
Breast, normal	(0.25 – 1) x 10^3	Sinkus et al.[156] Lorenzen et al.[157] Chen et al.[158] Griesenaur et al.[159]
Endothelium, aortic	(0.1 – 2) x 10^3	Sato et al.[160]
Breast, cancerous	~(1.5 – 3.5) x 10^3	Sinkus et al.[156] Lorenzen et al.[157] Chen et al.[158]
Liver	(12.88) x 10^3	Tay et al.[161]
Muscle tissue	(12 – 24) x 10^3	Engler et al.[162] Mathur et al.[163]
Articular cartilage	(0.4 – 200) x 10^6	Nemir and West[164]
Bone	(0.008 – 40) x 10^9	Nemir and West[164]

Table 1.1 – ECM stiffness varies across tissue types. Tissue stiffness measurements acquired from numerous methods are summarized here according to tissue type. The values displayed represent the tissue's measured Young's Modulus (E). Stiffness of tissue types varies drastically across the body to facilitate the differing physiological functions necessary for survival. This table is adapted from Gautier et al.[38]

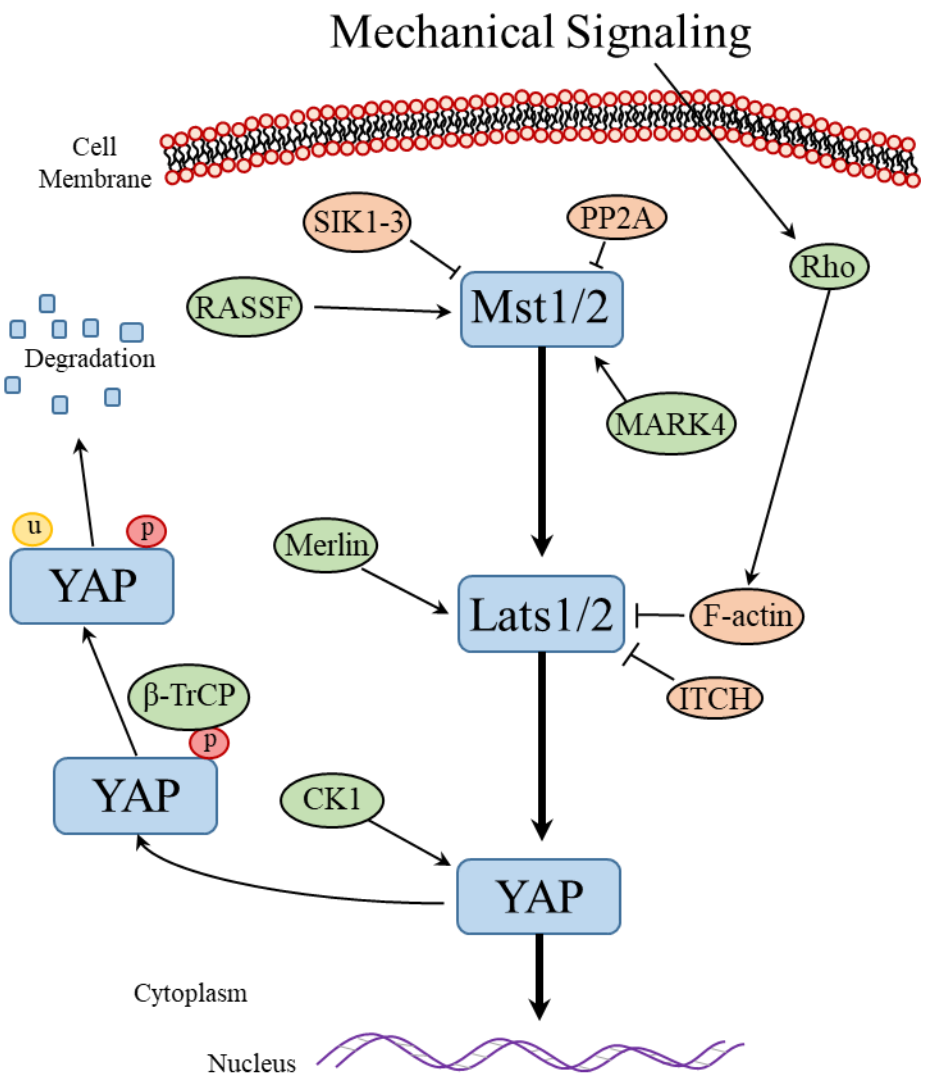

Fig. 1.1 – Canonical YAP pathway. YAP is a component of the Hippo signaling pathway. Phosphorylated (red) YAP remains in the cell cytoplasm where it is inactive. YAP is ubiquitinated (yellow) by β-TrCP which signals it for proteasome degradation. If Lats1/2 is inhibited and YAP remains unphosphorylated, it translocates to the nucleus where it acts as a transcriptional co-activator.

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Chapter 2: Characterization of Breast Cancer Cell Resistance to Chemotherapeutics in Alginate Hydrogels[2]

2.1 INTRODUCTION

The extracellular matrix (ECM) plays a critical role in the control of cell processes including cell survival, proliferation and apoptosis[1-3]. Cancer cells as well as normal cells integrate signals from ECM and may initiate different signaling programs in response to microenvironmental cues[4-6]. Understanding the contribution of mechanical signals from the 3D ECM in the tumor microenvironment is therefore important for predicting tumor progression and response to therapeutics.

There are many methods and platforms that can be used to grow and maintain cells as 3D cultures *in vitro*. One such method is to harvest matrix proteins such as collagens, laminins, or fibrins to construct a scaffold on which cells can grow. The primary advantage of using collagen and other native matrix proteins is their ability to closely mimic the fibril formation and a viscoelastic properties of their native environment[7]. Provenzano et al.[8] made use of this approach in studies of mouse mammary carcinoma cells cultured in dense collagen hydrogels to simulate mammographically dense breast tissue, a major risk factor for developing breast carcinomas[9-12]. However, this study also highlights one of the difficulties of using collagen hydrogels, namely that pure collagen hydrogels are typically of low stiffness (< 1 kPa) and stiffening collagen hydrogels comes at the cost of increasing overall collagen density or requires extensive chemical crosslinking.

Matrigel is a commercially available basement membrane extract consisting primarily of laminin, type IV collagen, entactin, and various growth factors[13]. The milieu of native matrix components and growth factors make it an appealing option for hydrogel cultures. However, Matrigel preparations are produced from basement membrane extracts from Engelbreth-Holm-

[2] Portions of this chapter were adapted from M. H. Joyce, C. Lu, E. R. James, R. Hegab, S. C. Allen, L. J. Suggs and A. Brock, "Phenotypic Basis for Matrix Stiffness-Dependent Chemoresistance of Breast Cancer Cells to Doxorubicin," *Front Oncol,* vol. 8, p. 337, 2018. A. B. and M. J. were responsible for the planning of the study and writing of the manuscript; C. L., E. J., M. J., R. H., and S. A.conducted the experiments; all authors were involved with the analysis of the manuscript.

Swarm mouse sarcoma tumors, meaning they are of tumorigenic origin and subject to significant batch-to-batch variation. This is an important factor for technologies and studies that require Good Manufacturing Processes (GMP) enforced by the Federal Drug Administration (FDA); it may also impact many studies that require a higher degree of precision in matrix composition.

Hyaluronic acid (HA) also falls in the native matrix component category. It is an anionic non-sulfated glycosaminoglycan distributed throughout connective, epithelial, and neural tissues. HA is an essential component of vertebrae ECM during growth, repair, and neoplasia[14-16]. It can be isolated from animal tissues or produced through microbial fermentation in *Escherichia coli*. Unmodified HA is not compatible with integrin-mediated cell adhesion, but rather facilitates cell adhesion through cell surface markers such as CD44 and CD168[17]. However, HA can be modified to present functional groups that allow for a broad range of crosslinking chemistries and can be further processed to form films (2D) or hydrogels (3D)[18]. Researchers have made use of this flexibility to investigate cellular mechanotransduction[19,20].

Non-native biological materials have also been shown to be suitable for mimicking ECM. Shin and Mooney[21] have previously used alginate-based hydrogels to mimic matrix stiffness conditions that would be physiologically relevant for myeloid leukemia cells. Using ionic cross-linking, it was feasible to produce hydrogels that model a range of microenvironments, from stiff bone marrow (Young's modulus E = 3 kPa) to low shear blood (E = 40 cP). The alginate-based system proved ideal for these conditions, as the stiffness of hydrogel cultures could be tuned by including increasing concentrations of calcium sulfate to achieve stiffer hydrogels. In these studies, hydrogels as soft as E = 75 Pa could be fabricated with the cross-linking agent (calcium sulfate), and fluid cultures (E = 40 cP) could be maintained when no cross-linking agent was added. This platform allowed Shin and Mooney to study matrix mechanics of myeloid leukemias subtypes in ECM mimicking diverse *in vivo* environments.

Synthetic polymers can also be used to facilitate 3D cultures. Polyethylene glycol (PEG), acrylamide, and custom polypeptides are examples of polymers that can be used to create hydrogels. Zustiak et al.[22] used collagen-coated polyacrylamide hydrogels to form hydrogels from

E = 1 kPa – 100 kPa. These hydrogels provide a very broad range of potential stiffness making them ideal for testing stiffness-dependence across many orders of magnitude. However, they must be coated with peptides or coupled with other matrix proteins to promote cell adhesion, and they are not a good representation of the fibrous networks that make up the ECM *in vivo*.

In this chapter, we utilize an alginate hydrogel model developed by Stowers et al.[23], to culture breast carcinoma cells in engineered microenvironments with specific stiffness properties. We investigate whether ECM stiffness in these hydrogels affects cell response to cytotoxic chemotherapeutic treatments. We explore two types of therapeutics, with different mechanisms of action, and compare their action in multiple human breast carcinoma and mouse mammary carcinoma models.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture

MCF7 cells were cultured in Minimum Essential Media (MEM, Life Technologies, REF: 11095-080, 89%) supplemented with Fetal Bovine Serum (FBS, Life Technologies, REF: 10437-028, 10%) and Penicillin/Streptomycin (P/S, Life Technologies, REF: 15070-063, 1%). Py2T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, REF: 10569-010, 89%) supplemented with FBS (Life Technologies, REF: 10437-028, 10%) and P/S (Life Technologies, REF: 15070-063, 1%). MDA-MB-231, TMEC, and M6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, REF: 10569-010, 94%) supplemented with FBS (Life Technologies, REF: 10437-028 5%) and P/S (Life Technologies, REF: 15070-063, 1%). All cultures maintained in a tissue culture incubator set to 37 °C with 5% CO_2. For 2D cultures, cells were grown as a monolayer in 12- (TrueLine, TR5000) or 96-well (Falcon, REF: 353072) plates. Studies done in 12-well plates have an n = 3 for each condition, while studies using 96-well plates have an n = 5 for each condition.

2.2.2 Hydrogel preparation

The system described in these studies is a Matrigel-alginate based system. Hydrogels were prepared by mixing the following ingredients in the order described with thorough mixing after addition of each ingredient: 4% alginate (1.6% final concentration, 40% total volume; ProNova UP MVG), 5 – 20 mM calcium carbonate (5% total volume), 100 mM NaCl + 1mM HEPES buffer (20% total volume), cells (5% total volume), 10 – 40 mM D-(+)-Gluconic acid δ-lactone (5% total volume), and Matrigel (25% total volume; VWR International). Once mixed, 50 µL of the gel solution was pipetted into each well of a 96-well plate and placed in an incubator (37 °C, 5% CO_2) for one hour to promote gelation. After gelation, 100 µL of media was added to each sample and placed back in an incubator. Optimal cell seeding density was found to be within the range of 8,000,000 cells/mL to 16,000,000 cells/mL; this seeding density ensured a sufficient amount of cells were cultured for data analysis without overcrowding the available growth area within hydrogels. For a 50 µL hydrogel this equates to approximately 25,000 to 50,000 cells per gel, respectively. Each condition will have an n = 3.

2.2.3 Rheometry

To determine hydrogel stiffness, a hydrogel solution was prepared following the protocol outlined in section 2.2.2, and approximately 50 µL of hydrogel solution was pipetted into an 8 mm diameter PDMS mold. Samples were then incubated overnight in a tissue culture incubator (37°C, 5% CO_2) before being measured on a Physica MCR 101 Rheometer using an 8 mm geometry (Anton Paar, Cat.#: 5681). Frequency sweep measurements were taken from 0.05 rad/s to 500 rad/s using Rheoplus (v3.40) software with 5% initial strain.

2.2.4 Dosing with chemotherapeutics

Samples were exposed to doxorubicin (Sigma-Aldrich, Cat.#: D1515) or paclitaxel (Sigma-Aldrich, Cat.#: T7402) for 48 h. A broad range (1 nM – 200 µM) of doses was used to determine the drug sensitivity.

2.2.5 Isolation of cells from hydrogels

To extract the cells cultured in hydrogels for analysis, each 50 µL gel was soaked in 100 µL of 50 mM sodium citrate (Fisher Scientific, Cat#: BP327) for 15 m at room temperature. Gels were then mechanically disrupted by pipetting until the alginate dissolved to a liquid solution. Each sample was transferred to a microcentrifuge tube and centrifuged at 600 x g for 10 m to pellet.

2.2.6 Measuring viability

Viability measures was assessed using acridine orange/propidium iodide (AOPI, Nexcelom, Cat#: CS2-0106) stain and MTS reagent (Promega, REF: G3580). Samples treated with AOPI were pelleted, resuspended in AccuMAX (Innovative Cell Technologies, Cat.#: AM-105), and mixed 1:1 with AOPI stain. Samples were then assayed using a Nexcelom Cellometer Vision. MTS assays were performed following the manufacturer's instructions. Briefly, all cell media was removed and replaced with MTS reagent mixed with media at 1:6 ratio, or 20 µL of MTS reagent with 100 µL of cell media per well of a 96-well plate. Samples were then incubated at 37 °C (5% CO_2) for 1 – 2 h before reading their absorbance at 490 nm on a plate-reader. The data gathered from both cell viability assays was fit to a sigmoid function in Microsoft Excel and used to calculate drug sensitivity, as measured by LD50 value.

2.3 RESULTS

2.3.1 An alginate hydrogel platform can be used to make ECM models of varying stiffness

This study sought to investigate how the ECM stiffness might affect breast cancer cell response to chemotherapeutic treatment. To this end, we employed an alginate hydrogel model developed by Stowers et al.[23]. This system was chosen because of the broad range of achievable stiffness, uniformity of stiffness throughout the hydrogel, ability to dynamically tune hydrogel stiffness (discussed in Chapter 3), and unique ability to isolate the effects of ECM stiffness on cells.

Stowers et al.[23] have shown the capability of this system to form hydrogels as soft as 40 Pa and as stiff as 20,000 Pa. The stiffness of this hydrogel system is driven by ionic calcium (Ca^{2+}) cross-linking G-blocks on alginate polymers. As additional cross-links are formed, the hydrogel will become progressively stiffer. The binding affinity of ionic calcium (Ca^{2+}) to these G-blocks is high enough[24] to cause almost immediate cross-linking. Attempting to mix ionic calcium (Ca^{2+}) with alginate will result in poor diffusion of calcium through the alginate and cause highly localized cross-linking. This system instead uses calcium carbonate ($CaCO_3$), which prevents immediate cross-linking by keeping ionic calcium (Ca^{2+}) bound to a carbonate (CO_3) molecule. To achieve cross-linking, glucono-δ-lactone (GDL) is added to the mixture with calcium carbonate and alginate. GDL is pH neutral, but hydrolyses in water to gluconic acid, which is subsequently buffered by the carbonate (CO_3) from the calcium carbonate ($CaCO_3$), thus allowing ionic calcium (Ca^{2+}) to bind to alginate G-blocks and form cross-links. This extends the cross-linking process such that it occurs over the span of hours rather than seconds, thus allowing gelation to occur evenly across the hydrogel.

Alginate is the major contributing factor to the hydrogel stiffness in this system, however alginate is bioinert[25] so Matrigel was added to provide cells with a substrate for the formation of cell-matrix adhesions and attachment sites. In this approach, cells are unable to directly rearrange the hydrogel and alter its stiffness. Such a system allowed us to further isolate the effects of stiffness on breast cancer cell resistance with limited dependence on cell adhesion type.

Hydrogels were prepared with 5 – 20 mM calcium carbonate and oscillatory shear stress rheometry was used to measure hydrogel stiffness. To collect stress and strain measurements, a hydrogel solution was prepared following the protocol outlined in section 2.2.2. Briefly, approximately 50 µL of hydrogel solution was pipetted into an 8 mm diameter PDMS mold. Samples were then incubated overnight in a tissue culture incubator (37°C, 5% CO_2) before being measured on a Physica MCR 101 Rheometer using an 8 mm geometry (Anton Paar, Cat.#: 5681). Frequency sweep measurements were taken from 0.05 rad/s to 500 rad/s using Rheoplus (v3.40) software with 5% initial strain. The elastic modulus was calculated for each frequency measured

within this range. Our results show that we were able to make gels ranging from 450 – 2,000 Pa (5 mM = 228 Pa, n = 3; 10 mM = 910 Pa, n = 1; 15 mM = 1,368, n = 8; 20 mM = 1,958 Pa, n = 5; **Fig. 2.1a**) by varying the concentration of calcium carbonate added to the initial gel mixture.

2.3.2 Characterization of mammary epithelial carcinoma cells to chemotherapeutic response in monolayer cultures

We began our study by challenging breast cancer cell lines with two clinically relevant chemotherapeutics – doxorubicin and paclitaxel. MDA-MB-231 and MCF7 cells were cultured as a monolayer on tissue culture plastic (2D) and challenged with each drug as a single agent (**Fig. 2.1b**). After 48 h exposure the chemotherapeutic was removed and replaced with MTS reagent mixed into culture medium at a 1:6 ratio. Samples were incubated at 37C for 2 h, to allow for sufficient metabolism of the MTS reagent, and immediately placed on a plate-reader to measure 490 nm absorbance. These readings were used to calculate cell viability and fit to a sigmoidal function to determine the 50% lethal dose (LD50) for each drug-cell combination. The results outlined in **Fig. 2.2** show that MDA-MB-231 cells were more resistant to doxorubicin (LD50 = 26.6 μM, **Fig. 2.2a**) than paclitaxel (LD50 = 94 nM, **Fig. 2.2b**). MCF7 cells showed a similar response with cells being more resistant to doxorubicin (LD50 = 4.4 μM, **Fig. 2.2c**) than paclitaxel (LD50 = 126 nM, **Fig. 2.2d**).

Given that these cells lines had similar responses to paclitaxel but not doxorubicin when cultured as monolayers (2D), we expanded our study to evaluate the response of additional cell lines to doxorubicin (**Fig. 2.3**). TMEC, M6, M6c, and Py2T cell lines were all cultured as a monolayer and challenged for 48 h with doxorubicin. We observed that the MDA-MB-231 and Py2T cell lines had a significant advantage over the MCF7, M6, and M6c cell lines when exposed to doxorubicin.

2.3.3 Characterization of mammary epithelial carcinoma cells to chemotherapeutic response in alginate hydrogel cultures

Our experiments with monolayer cultures have shown that mammary epithelial carcinoma cell lines can have differing responses to treatment with doxorubicin. It has previously been shown that growing cells in 3D cultures can increase their resistance to chemotherapeutic drugs[26-28]. We sought to explore this with mammary epithelial carcinoma cell lines cultured in the previously described alginate hydrogel system. Samples were cultured for six days in either 450 Pa (**Fig. 2.4**) or 2,000 Pa (**Fig. 2.5**) hydrogels before treatment with doxorubicin; this was done to allow cultures plenty of time to form microstructures before exposure to treatment. Our goal was to determine if cells grown in 3D cultures would respond differently to the chemotherapeutics, and if so, to quantify these differences. We observed that chemoresistance of MDA-MB-231 cells to doxorubicin was 3-fold higher in the stiff ECM environment (LD50 = 10 μM in 450Pa hydrogel cultures vs. LD50 = 32 μM in 2,000Pa cultures; $p = 0.002$; **Fig. 2.6a,c**). MCF7 cells did not display any significant ($p = 0.134$; **Fig. 2.6b,d**) differences in resistance across substrates of increasing stiffness.

2.3.4 Acute responses to changes in ECM stiffness increase resistance to doxorubicin

Following our experiments that showed differing responses of mammary carcinoma cell lines to doxorubicin in alginate hydrogels, we questioned if the time a cell was exposed to their ECM would affect their response to treatment with doxorubicin. Our hypothesis was that cells exposed to stiffer ECM for longer would have increased resistance to doxorubicin. To test this hypothesis, we cultured MDA-MB-231 (**Fig. 2.7a**) and MCF7 (**Fig. 2.7b**) cells in 450 Pa or 2,000 Pa alginate hydrogels for 4 (3 days + 24 h), 6 (3 days + 72 h), or 8 (3 days + 120 h) days before challenging them with a 48 h exposure to doxorubicin.

Our results showed that MDA-MB-231 cells cultured in either 450 Pa or 2,000 Pa for 4 days had no significant difference in resistance to doxorubicin ($p = 0.822$, **Fig. 2.7a**). However, after 6 days ($p = 0.002$, **Fig. 2.7a**) and 8 days ($p = 0.0005$, **Fig. 2.7a**) MDA-MB-231 cells cultured in 2,000 Pa hydrogels showed an increase in resistance to doxorubicin compared to similar cultures

in 450 Pa hydrogels. MCF7 cells also showed no significant difference in doxorubicin resistance at 4 days ($p = 0.575$, **Fig. 2.7b**), 6 days ($p = 0.134$, **Fig. 2.7b**), or 8 days ($p = 0.333$, **Fig. 2.7b**) when comparing 450 Pa cultures to 2,000 Pa cultures. However, there was a significant decrease in resistance for 450 Pa cultures ($p = 0.001$, **Fig. 2.7b**) and 2,000 Pa cultures ($p = 0.046$, **Fig. 2.7b**) between the 4 day and 6 day time-point. No significant difference was found between 450 Pa cultures ($p = 0.064$, **Fig. 2.7b**) and 2,000 Pa cultures ($p = 0.965$, **Fig. 2.7b**) between the 6 day and 8 day time-point.

Viability assays were performed on cells that were cultured in 450 Pa or 2,000 Pa hydrogels at 48 h post-seeding and 1 week post-seeding (**Fig. 2.8a**). The results were compared to similar cells grown on tissue culture plastic, and we found that the total viability was similar for both MDA-MB-231 and MCF7 cells grown in all conditions. The culture media of these samples was also collected and measured for pH at the same time as viability testing (**Fig. 2.8b**). We found all pH measures to be within acceptable ranges given the values of the 2D (control) samples and previous findings in the literature stating that a malignant tumor's microenvironment can range from pH 6.5 – 6.9[29].

2.4 Discussion

Using the alginate hydrogel model described by Stowers et al.[23], we optimized conditions to generate hydrogels mimicking the ECM stiffness of biologically healthy (~450 Pa) to early stage breast tumors (~2,000 Pa)[30]. We used this experimental model to determine how ECM mimicking both normal and early stage tumor tissue affects mammary epithelial carcinoma cell response to treatment with chemotherapeutics. Initially, we challenged samples with doxorubicin and paclitaxel. Doxorubicin, also known by the trade name Adriamycin, is a common chemotherapeutic used to treat neoadjuvant breast cancer. It works by intercalating into DNA and inhibiting progression of topoisomerase II after it has broken the DNA double helix for replication[31]. This prevents the DNA strands from resealing, thereby stopping DNA replication and ultimately leading to cell death after accumulation of enough unrepaired fragments. Paclitaxel

(sold under the trade name Taxol) is a member of the taxane family of drugs which work by disrupting microtubule function[32]. Microtubules in the mitotic spindle are normally highly dynamic, growing and shrinking to facilitate segregation of chromosomes to daughter cells during mitosis[33]. Paclitaxel functions as a microtubule-stabilizing agent, binding to β-tubulin near the M loop and increasing affinity for tubulin molecules, ultimately suppressing microtubule dynamics[34,35]. It has been shown to cause cell death by trapping cells in the metaphase/anaphase transition of mitosis, and thus acts preferentially on cancer cells due to their increased mitotic activity[36,37].

Our results showed very similar LD50s for both MDA-MB-231 and MCF7 cells to paclitaxel in 2D cultures, but MDA-MB-231 cells had a 6-fold higher LD50 to doxorubicin compared to MCF7 cells. The differing response of these two mammary epithelial carcinoma lines to the same drug led us to challenge a panel of mammary epithelial carcinoma lines. This panel of cells was cultured both as monolayers (2D) and in hydrogels (3D) and challenged with doxorubicin. We found that cells displaying a mesenchymal phenotype (MDA-MB-231, Py2T-LT, TMEC) showed a stiffness-dependent increase in resistance to doxorubicin, whereas cell types displaying epithelial phenotypes (MCF7, M6, M6c) did not. This finding is similar to myeloid leukemia subtypes described by Shin & Mooney[38]. They cultured two myeloid leukemia cell lines in alginate hydrogels of varying stiffness and functionalized with varying ligands and proceeded to challenge cultures with chemotherapeutics commonly used to treat myeloid leukemia. Their results showed three subtypes of myeloid leukemia – class I being cells that were ligand sensitive, class II being cells that were ligand and matrix stiffness sensitive, and class III being cells that were mechanics independent. Our results indicate that the mesenchymal lines we tested would fall under the class II subtype, with the epithelial lines falling under the class III subtype. This is further supported by Lovitt et al.[39] who showed in their 2018 study that functional inhibition of β1-integrin signaling in 3D cultures of MDA-MB-231 cells has a dose-dependent increase in sensitivity to doxorubicin.

We next sought to determine if the duration of exposure to ECM stiffness would affect resistance to doxorubicin. By culturing cells for varying periods of time in defined ECM environments, we explored the temporal dynamics of ECM-stiffness mediated responses. Our results indicate that there is an acute response to ECM changes, but this response levels out after approximately 4 days in the hydrogel. This area is currently understudied, but is an important consideration when screening for drug sensitivity or efficacy.

2.5 CONCLUSIONS

Here we have shown that mammary epithelial carcinoma cell lines will respond differently when cultured in hydrogels (3D) as opposed to monolayers. Our results shed light on the importance of ECM stiffness and duration of exposure to ECM stiffness when evaluating drug sensitivities of mammary epithelial carcinoma cells. We believe that giving more attention to ECM stiffness and exposure to ECM stiffness prior to treatment will lead to more accurate simulations of drug efficacy.

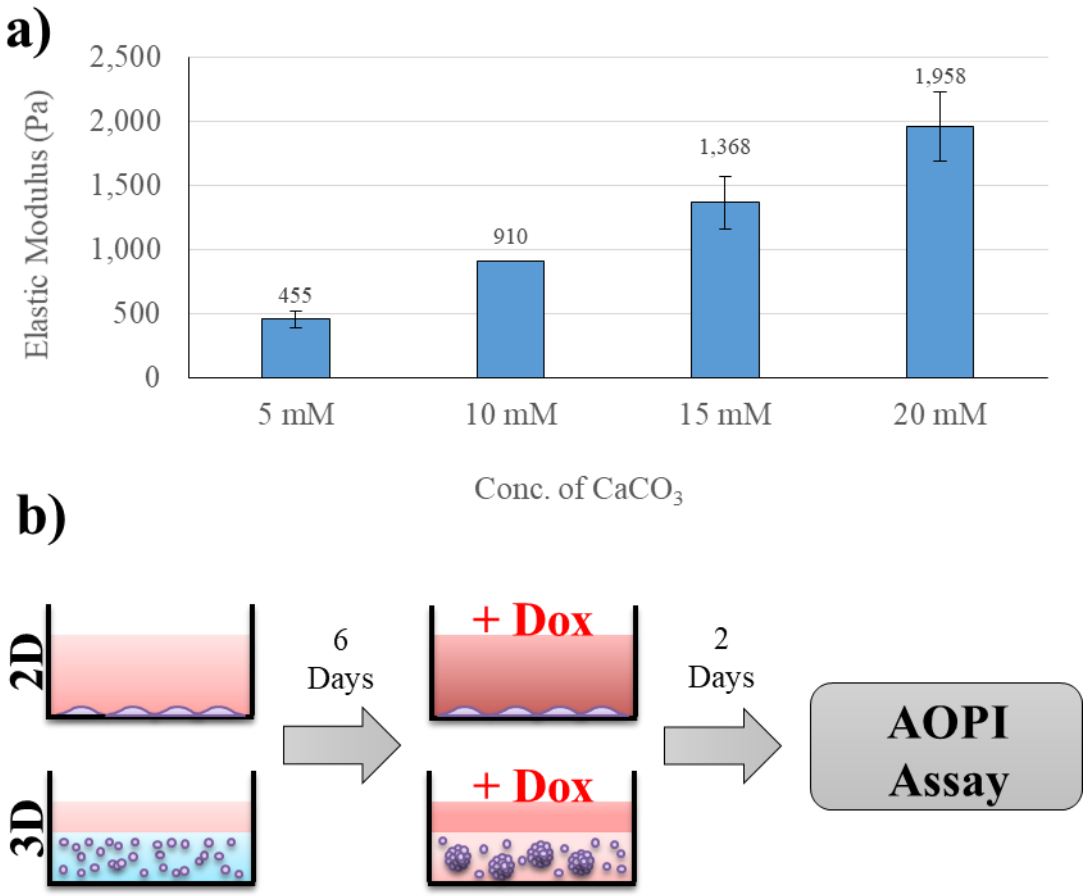


Fig. 2.1 – Cells were cultured in hydrogels of varying stiffness before treatment with doxorubicin. a). Hydrogel stiffness was determined by calculating Young's modulus from frequency sweep measurements obtained from a rheometer**.** Error bars represent a 95% confidence interval as determined by a Student's t-test distribution. The experimental protocol is outlined in **b)**. Briefly, cells were seeded onto tissue culture plastic or into hydrogels that ranged in stiffness from 450 to 2,000 Pa. After 6 days in culture, samples were exposed to doxorubicin for 48h and cell viability was determined using AOPI staining.

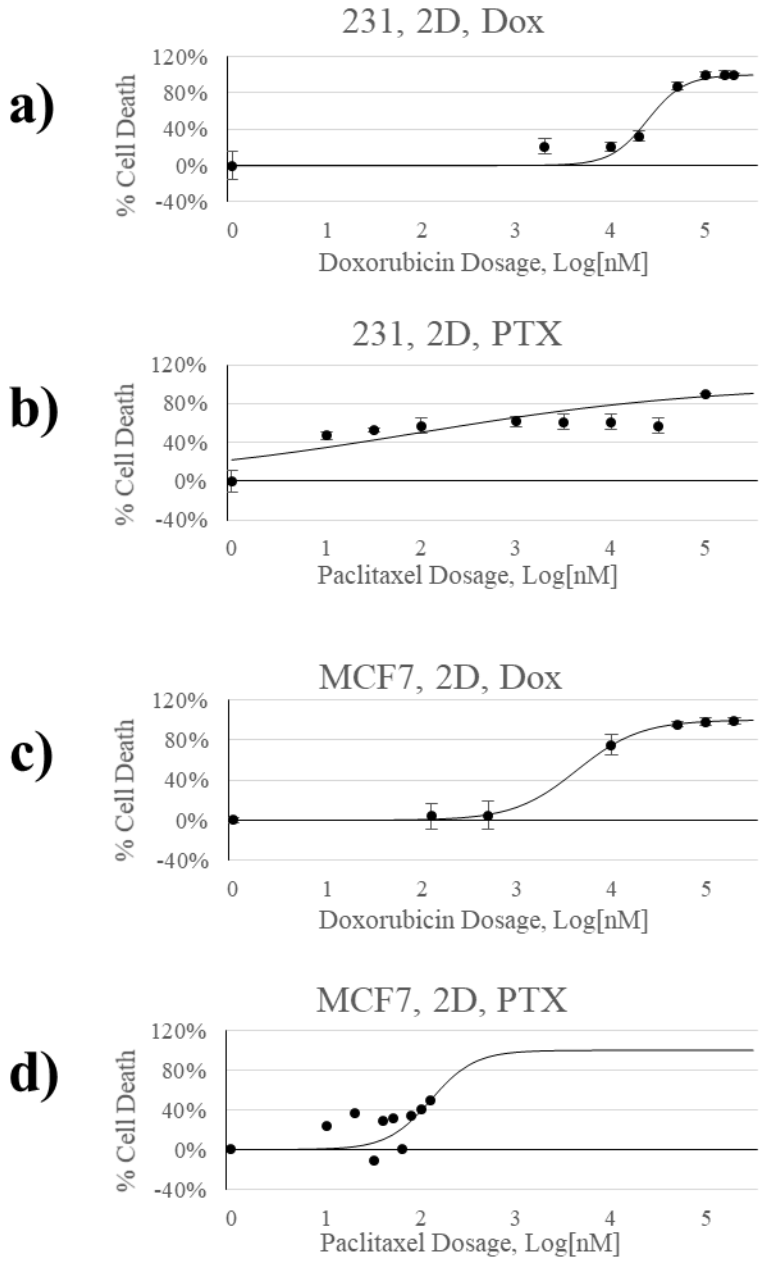


Fig. 2.2 – Cells were cultured as a 2D monolayer and exposed to doxorubicin or paclitaxel. MDA-MB-231 and MCF7 cells were cultured as a 2D monolayer and exposed to doxorubicin or paclitaxel for 48 h. **a)** MDA-MB-231 cultures were found to be more resistant to doxorubicin (LD50 = 26.6 μM) than **b)** paclitaxel (LD50 = 94 nM). **c)** MCF7 cultures were also found to be more resistant to doxorubicin (LD50 = 4.4 μM) than similar **d)** MCF7 cultures treated with paclitaxel (LD50 = 126 nM).

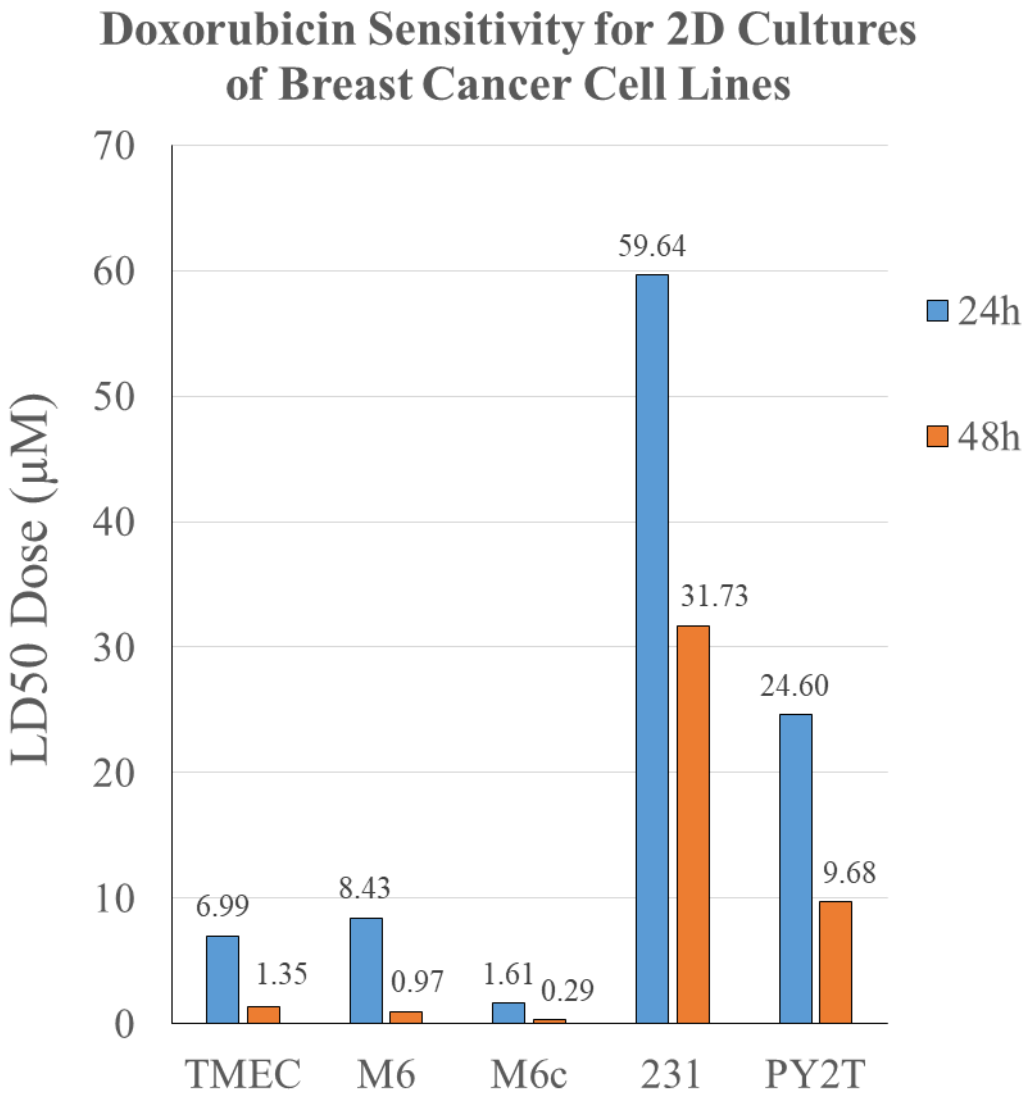


Fig. 2.3 – Additional cell lines were cultured as a 2D monolayer and challenged with doxorubicin. Additional mammary epithelial carcinoma cell lines were cultured as 2D monolayers and exposed to doxorubicin for 24 and 48 h before assaying for cell viability. MDA-MB-231 and Py2T cell lines were found to be more resistant to doxorubicin than the TMEC, M6, or M6c cell lines.

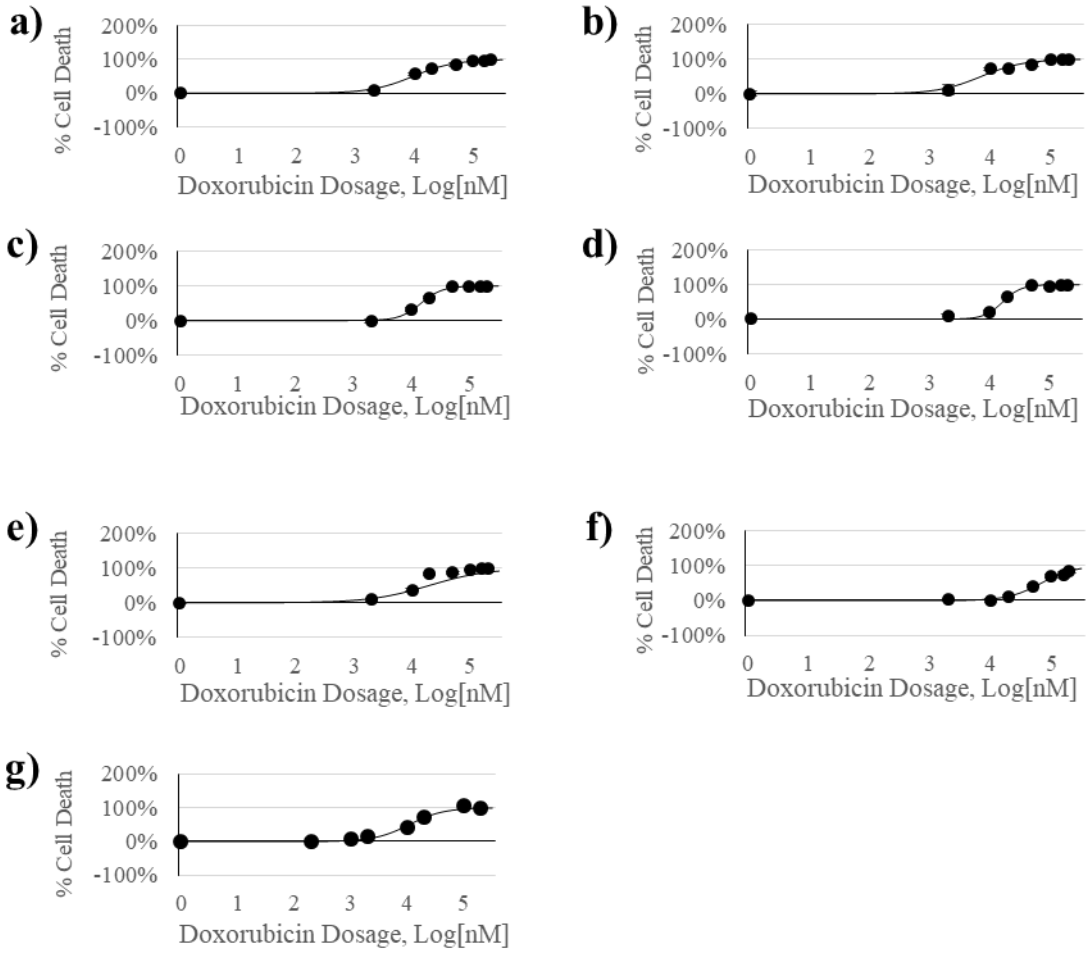


Fig. 2.4 – Dose response curves of **a)** MDA-MB-231, **b)** MCF7, **c)** M6, **d)** M6c, **e)** TMEC, **f)** Py2T, **g)** Py2T-LT following 48h exposure to Doxorubicin. All cells were cultured in 450 Pa alginate-Matrigel hydrogels for 8 days before being exposed to Doxorubicin. Live and Dead cells were counted using a Nexcelom Cellometer with AOPI stain.

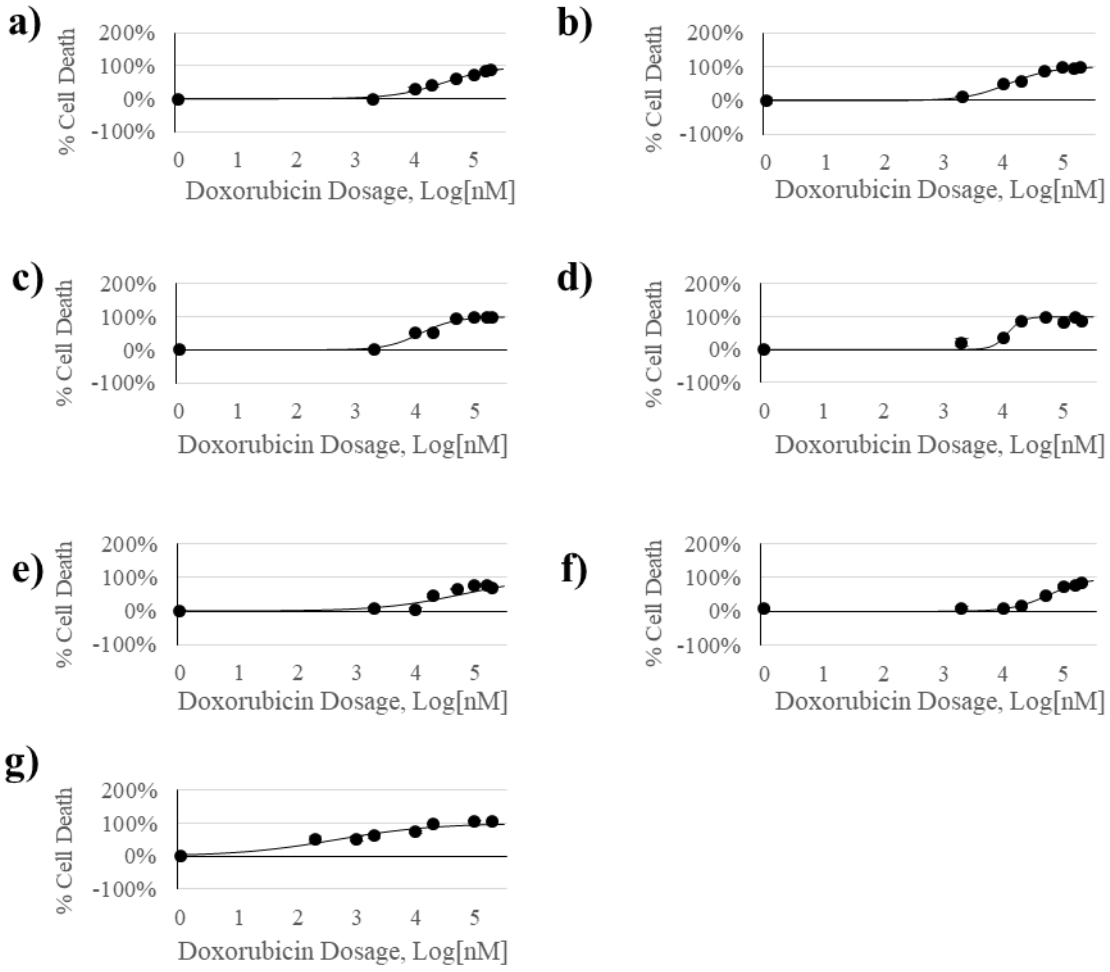


Fig. 2.5 – Dose response curves of **a)** MDA-MB-231, **b)** MCF7, **c)** M6, **d)** M6c, **e)** TMEC, **f)** Py2T, **g)** Py2T-LT following 48h exposure to Doxorubicin. All cells were cultured in 2,000 Pa alginate-Matrigel hydrogels for 8 days before Doxorubicin exposure. Live and Dead cells were counted using a Nexcelom Cellometer with AOPI stain.

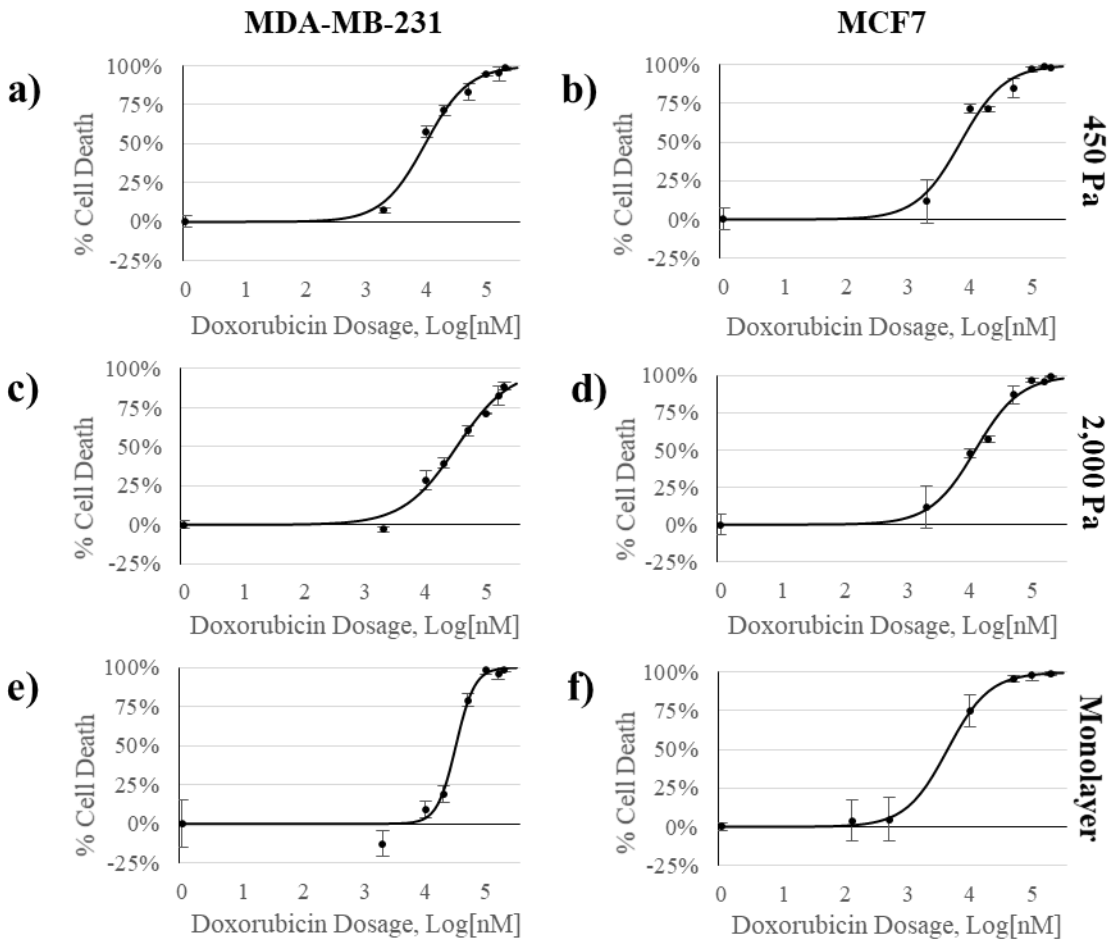


Fig. 2.6 – 231 cells have a stiffness-dependent resistance to doxorubicin. Dose response curves of 231 and MCF7 cells cultured in **a,b)** 450 Pa hydrogel, **c,d)** 2,000 Pa hydrogel, and **e,f)** 2D monolayer following 48h exposure to Doxorubicin. Percent cell death was determined by staining samples with AOPI and counting live cells using a Nexcelom Cellometer.

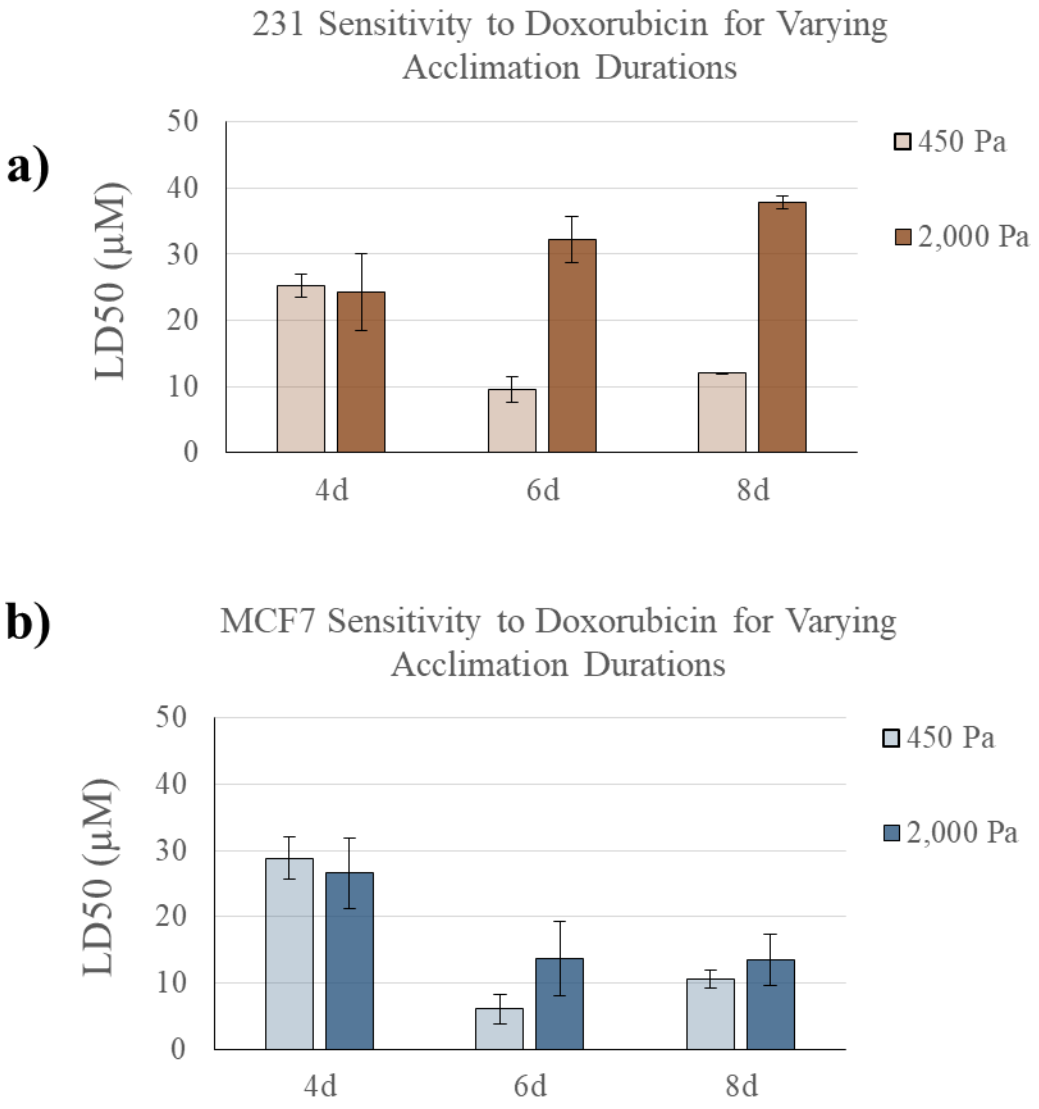
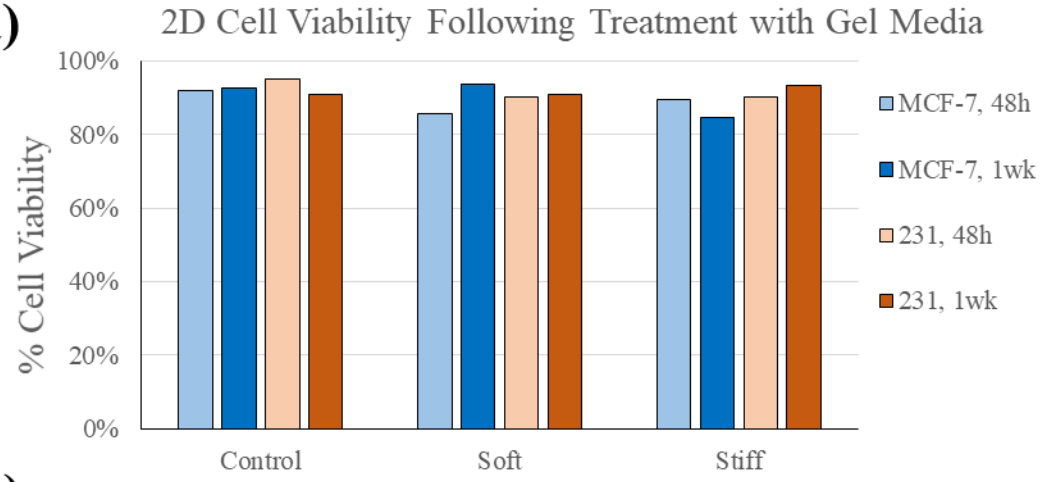


Fig. 2.7 – Shorter acclimation time gives temporary increase in doxorubicin resistance. LD50 values of **a)** MDA-MB-231 and **b)** MCF7 cells cultured in 450 Pa hydrogel or 2,000 Pa hydrogel for 24, 72, or 120 h before a 48 h exposure to doxorubicin. Percent cell death was determined by staining samples with AOPI and counting live cells using a Nexcelom Cellometer.

a)



b)

Cell Type	Media	pH (48h)	pH (1wk)
MCF7	Control	8.28	7.85
	450 Pa	7.85	7.87
	2,000 Pa	6.95	7.95
231	Control	8.09	7.6
	450 Pa	7.9	7.62
	2,000 Pa	7.16	7.95

Fig. 2.8 – Inclusion of D-(+)-Gluconic acid δ-lactone in hydrogel mixture does not adversely affect cell viability. Cells were cultured as a monolayer (2D) on tissue culture plastic with culture media supplemented with concentrations of calcium carbonate and D-(+)-Gluconic acid δ-lactone equal to those found in 450 Pa and 2,000 Pa hydrogel mixtures. Samples were seeded with the supplemented media and subsequently fed with normal culture media every other day for the remainder of the experiment to mimic the conditions that cells seeded in hydrogels would be exposed to.

After 5 days in culture, **a)** cell viability was assayed using acridine orange/propidium iodide (AOPI) staining, and **b)** the media was removed from samples to measure pH values. Estrella et al.[40] notes that the pH of a malignant tumor's microenvironment can range for pH 6.5 – 6.9. MCF10A cells cultured in presence of the 2,000 Pa hydrogel mixture conditions are the only samples to drop below this pH value causing almost complete cell death. The remaining MCF7 and 231 cell lines showed only slight deviations in pH from control conditions (normal culture media for the duration of the experiment), though all of these values fall within an acceptable range, and minimal cell death compared to control conditions.

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Chapter 3: Characterization of Breast Cancer Cell Response to Chemotherapeutics in Dynamic Stiffened Hydrogels[3]

3.1 INTRODUCTION

The ECM is a major component of the cellular microenvironment that facilitates many basic cellular functions[1] due to its dynamic and transmutable nature[2]. Cells alter their surrounding ECM by changing its composition, through synthesis or degradation of ECM components, or architecture, through cross-linking via covalent and noncovalent modifications[2]. Synthesis of ECM components occurs locally. For example, in developing mammary glands, the fibroblasts surrounding epithelium will synthesize and deposit collagen I, one of the most abundant ECM proteins in mammary gland tissue[3]. Degradation of ECM components is largely directed by the matrix metalloproteinases (MMPs)[4,5] with help from adamalysins[6-9], meprins[10], Ser proteases[11,12], cathepsins[13], heparanases[4], and sulphatases[4]. Cells will also align collagen fibers and other structural ECM components through integrin-ECM interactions. It is this interplay of ECM degradation, synthesis, and rearrangement that maintains the ECM in a dynamic state and forms the basis for a reciprocal relationship between cells and the matrix microenvironment.

Numerous diverse experimental approaches have been developed to recreate specific aspects of the ever-changing ECM. Vincent et al.[14] describe a method whereby cells are seeded on a coverslip island in the middle of a PDMS hydrogel to simulate a change in substrate stiffness and composition. During preparation of the hydrogel, the coverslip is placed on top of the partially gelled PDMS, allowing a small amount of PDMS to creep over the edge of the coverslip and form an interface between the glass and hydrogel[14,15]. This enabled the researchers to study how cells respond to changes in substrate stiffness and the cellular mechanics that are involved with these processes.

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[3] Portions of this chapter were adapted from M. H. Joyce, C. Lu, E. R. James, R. Hegab, S. C. Allen, L. J. Suggs and A. Brock, "Phenotypic Basis for Matrix Stiffness-Dependent Chemoresistance of Breast Cancer Cells to Doxorubicin," *Front Oncol,* vol. 8, p. 337, 2018. A. B. and M. J. were responsible for the planning of the study and writing of the manuscript; C. L., E. J., M. J., R. H., and S. A.conducted the experiments; all authors were involved with the analysis of the manuscript.

Guvendiren & Burdick[16] developed a methacrylated hyaluronic acid (MeHA) hydrogel system capable of dynamically stiffening through UV irradiation. This system allows for the initial formation of a hydrogel through addition of dithiothreitol (DTT), which serves to crosslink some of the methacrylates present in the MeHA solution. Additional stiffening comes through UV irradiation of a photoinitiator (Irgacure 2959) that releases free radicals which crosslink the remaining methacrylate groups. Using this system, the researchers were able to form hydrogels ranging in stiffness from ~3 kPa to ~100 kPa through addition of DTT only, and used UV-induced stiffening to dynamically stiffen hydrogels from ~3 kPa to ~30 kPa.

Gillette et al.[17] combined two natural polymers – collagen I and alginate – to design a hydrogel system that could be dynamically stiffened or softened through the addition of calcium chloride (CaCl) or sodium citrate, respectively. Collagen and alginate were combined to form the initial hydrogel within a PDMS mold placed on a glass slide; a thin aluminum oxide or cellulose membrane was placed over the hydrogel to allow ions to pass through, but prevents passage of larger alginate polymer chains. To dynamically stiffen hydrogels after the initial seeding, a 60 mM calcium chloride solution was pipetted onto the membrane to diffuse through to the hydrogel and induce crosslinking of alginate polymers. Similarly, to soften the hydrogel, a 5% w/v sodium citrate solution can be washed over the membrane to allow for diffusion through to the alginate hydrogel and chelation of the calcium crosslinking alginate polymers.

These are but a few of the methods that researchers have employed to study how a dynamic ECM will impact cellular responses. While each of these methods has its own strengths and limitations, they, as well as others, have aided the discovery of therapeutic targets and treatment options to mitigate tumor progression.

Tumors stiffen as they progress, and this presents issues for treatment options by limiting drug diffusion to targeted tissues and exerting environmental pressures that facilitate further disease progression. Potential therapeutic options that are currently being explored include use of collagenase, collagen[18-20] or hyaluronic acid[21-24] synthesis inhibitors, and integrin inhibitors[25]. These methods have traditionally been employed as a means to soften tissue and thereby increase

drug diffusivity; however studies focused on cell-ECM interactions also suggest that such changes in the cells' microenvironment may be sufficient to transition tumor cells to a more drug sensitive state[25,26].

Previous studies observed how cells adapt to changes in their ECM, but the system developed by Stowers et al.[27] is uniquely adapted to study the effects of dynamic ECM stiffness. Lipsomes loaded with ionic calcium and gold nanorods (20% total volume) are added to 4% alginate (1.6% final concentration, 40% total volume; ProNova UP MVG), 5 – 20mM calcium carbonate (5% total volume), cells (5% total volume), 10 – 40mM D-(+)-Gluconic acid δ-lactone (5% total volume), and Matrigel (25% total volume; VWR International) (as in chapter 2) then stimulated with NIR light to induce further hydrogel stiffening. This occurs because exposure to NIR light causes the gold nanorods to undergo surface plasmon resonance and heat the lipid layer close to its gel-to-fluid phase transition at 41°C, allowing the ionic calcium to leak out of liposomes and form additional cross-links with nearby alginate polymers. We therefore sought to use this platform to investigate our hypothesis that the physical forces from a stiffening microenvironment would increase breast cancer resistance to chemotherapeutic treatment. In addition, we hypothesized that varying the exposure time of cells to stiffened ECM would attenuate this stiffness-dependent drug response. The ability to dynamically stiffen the ECM surrounding breast cancer cell cultures in situ, without requiring cells to be removed from a specific hydrogel environment and reseeded in a different environment, made this alginate hydrogel system an ideal platform to test our hypotheses.

3.2 Materials and Methods

3.2.1 Liposome preparation

Liposomes were prepared using the interdigitation-fusion method described by Ahl et al.[28]. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti, 850355P/C) was diluted in chloroform (Fisher Scientific, CAS: 67-66-3) at 25 mg/mL and rotary evaporation (150 mbar vacuum, ~60 rpm, 55 °C, 15 m) was used to coat a round-bottom flask with thin layers of lipids.

After 15 m incubation on the rotary evaporator, the newly formed lipid cake was placed in a desiccator overnight to ensure complete evaporation of any residual chloroform. The lipid cake was rehydrated with 2 mL ultra-pure water and placed on a rotator for 30 m to ensure hydration of the entire lipid cake. The solution was then sonicated via sonic probe (60% amplitude for 10 m) to form small unilaminar vesicles. At this point, the lipid solution was passed through a 0.22 µm filter (Millipore, SLGS033SB) and 424 µL of 100% ethanol (Fisher Scientific, CAS: 64-17-5) was added to form interdigitated sheets. Gold nanorods and 500mM calcium chloride (Sigma-Aldrich, C1016) was added and allowed to incubate at 55 °C for 2 hours with gentle agitation every 30 m to ensure encapsulation of cargo into newly forming liposomes. After incubation, a series of washes with 300 mM sodium chloride (Fisher Scientific, 7647-14-5) plus 1 mM HEPES (Sigma-Aldrich, H3375) was performed to remove any free-floating nanorods or calcium.

3.2.2 Hydrogel preparation

Hydrogels were prepared using similar methods described in chapter 2, with the addition of liposomes to the gel mixture. The following were combined in the order listed, with thorough mixing after each new item added to the gel mixture: 4% alginate (Pronova UP MVG; 40% total volume, 1.6% final concentration), 5 – 20 mM $CaCO_3$ (5% total volume), liposomes loaded 500 mM $CaCl_2$ plus AuNRs (20% total volume), cells (8 million – 16 million cells/mL; 5% total volume), 10 – 40 mM D-(+)-Gluconic acid δ-lactone (Sigma-Aldrich, G4750; 5% total volume), and Matrigel (VWR International, 47743-715; 25% total volume). 50 µL of the final mixture was pipetted into each well of a 96-well plate (Falcon, REF: 353072) and placed in an incubator (37°C, 5% CO_2) for 1 h to promote gelation. 100 µL of culture media was added to each well and cultures were returned to the incubator for cell growth and maintenance.

3.2.3 Dynamic stiffening of alginate hydrogels

Prior to exposing samples to near infrared light, all media was aspirated from each sample to minimize light scattering. A Lasermate (IML808-2500FLAM4A) set at 2.0 W was used to

irradiate samples for 45 s each. After irradiating the final sample, 100 µL of fresh culture media was added to each culture well and the samples were returned to the cell incubator (37 °C, 5% CO_2) for routine growth and maintenance.

3.2.4 Statistical analysis

A two-tailed Student's t-test assuming unequal variance was used to compare samples, and a p-value of less than 0.05 was used to determine statistical significance. Microsoft Excel Solver was used to fit cell viability data to a sigmoidal function to generate LD50 curves.

3.3 RESULTS

Cells were cultured in an alginate-Matrigel hydrogel platform, as described in Stowers et al.[27], which uses calcium-loaded liposomes to drive cross-linking following exposure to near-infrared (NIR) light. Cells were initially cultured in hydrogels for 3 days to begin formation of spheroid micro-structures (**Fig. 3.1a**). Hydrogels were stiffened via NIR triggered cross-linking (**Fig. 3.1b,c**), and cells were cultured for 3 additional days before treatment with doxorubicin for 48 h (**Fig. 3.1a**). MDA-MB-231 cultures that were grown in dynamically stiffened hydrogels had a significant decrease in sensitivity to doxorubicin for cultures that were stiffened from 450 Pa to 1,600 Pa (LD50 = 10 µM in 450 Pa static cultures vs. LD50 = 80 µM in cultures stiffened from 50 Pa to 1,600 Pa; p = 0.004; **Fig. 3.2a**) as well as those stiffened from 2,000 Pa to 3,000 Pa (LD50 = 32 µM in 2,000 Pa static cultures vs. LD50 = 185 µM in cultures stiffened from 2,000 Pa to 3,000 Pa, p = 0.014) compared to their static hydrogel counterparts (**Fig. 3.2a**). This relationship was not observed in MCF7 (**Fig. 3.2b**); there was no statistically significant difference in sensitivity to doxorubicin for cultures stiffened from 450 Pa to 1,600 Pa (LD50 = 6 µM in 450 Pa static cultures vs. LD50 = 13 µM in cultures stiffened from 450 Pa to 1,600 Pa; p = 0.143) or 2,000 Pa to 3,000 Pa (LD50 = 12 µM in 2,000 Pa static cultures vs. LD50 = 17 µM in cultures stiffened from 2,000 Pa to 3,000 Pa; p = 0.492) when compared to static cultures.

The time between dynamic stiffening of hydrogel cultures and treatment with doxorubicin was varied to determine if cells undergo adaptation to stiffened ECM that may alter drug sensitivity. Cells were cultured in hydrogels for 3 days to allow formation of micro-structures before being exposed to NIR to induce dynamic stiffening. Cultures were then maintained 24, 72, or 120 h in the stiffened ECM before treatment with doxorubicin. Resistance to doxorubicin peaked in MDA-MB-231 cells that were treated 72 h post-stiffening with an LD50 of 80 μM for cultures stiffened from 450 to 1,600 Pa and 185 μM for cultures stiffened from 2,000 to 3,000 Pa (**Fig. 3.2a**). By 120 h post-stiffening, this increase in resistance dropped to 87 μM for cultures stiffened from 450 to 1,600 Pa ($p = 0.323$) and 71 μM for cultures stiffened from 2,000 to 3,000 Pa ($p = 0.021$). We hypothesize that the stiffening from 2,000 Pa to 3,000 Pa elicits an acute response from MDA-MB-231 cells that results in an increased resistance to doxorubicin, which is shown at the 72 h acclimation time-point. However, our data suggests this response equilibrates by the 120 h acclimation time-point. Sensitivity of 231 to doxorubicin was greatest at 24 h post-stiffening with LD50 measures of 5 μM for cultures stiffened from 450 to 1,600 Pa and 3 μM for cultures stiffened from 2,000 to 3,000 Pa.

MCF7 cultures behaved inversely to their MDA-MB-231 counterparts (**Fig. 3.2b**). Peak resistance for MCF7 cultures was observed following 24 h acclimation in the stiff gels, with an LD50 of 26 μM for cultures stiffened from 450 to 1,600 Pa and 16 μM for cultures stiffened from 2,000 to 3,000 Pa. Resistance progressively decreased to LD50 values of 13 μM ($p = 0.154$) and 8 μM ($p = 0.104$) for cultures stiffened from 450 to 1,600 Pa and 17 μM ($p = 0.834$) and 7 μM ($p = 0.072$) for cultures stiffened from 2,000 to 3,000 Pa at 72 h and 120 h acclimation, respectively. However, when comparing across different lengths of acclimation periods, these decreases in resistance were not statistically significant.

Dynamic stiffening had a significant difference for every acclimation duration tested with MDA-MB-231 cultures as determined by a Student's t-test with $p < 0.05$ denoting significance. This suggests that the duration of exposure to a particular set of ECM microenvironmental stiffness conditions contributes to resistance to doxorubicin in these cells.

3.4 DISCUSSION

Herein we describe how we used an alginate system coupled with calcium-loaded liposomes to simulate the stiffening of the tissue microenvironment that is observed in breast cancer progression. Similar to our results from chapter 2, we observed that MDA-MB-231 cells have increased resistance to doxorubicin following dynamic stiffening of their ECM. Slight increases in resistance were seen in similar MCF7 cultures, however these results were not shown to be statistically significant. We believe that similar mechanisms govern the stiffness-dependent resistance seen in dynamically stiffened hydrogels and static hydrogels, namely, those that underlie phenotypic expressions driving the mesenchymal and epithelial characteristics of each cell line. Interestingly, we found that dynamically stiffening hydrogels increased the resistance of MDA-MB-231 cells more than just placing them in a static hydrogel of similar stiffness alone (**Fig. 3.2a**).

To further investigate this effect, we varied the time between dynamically stiffening the hydrogel and subsequent exposure to the chemotherapeutic agent doxorubicin (**Fig. 3.1a**). We observed that MDA-MB-231 cultures were more sensitive to doxorubicin when only given 24 h to acclimate to the dynamically stiffened hydrogels. In sharp contrast, their resistance to doxorubicin peaked when given 72 h acclimation time between stiffening and drug exposure. MDA-MB-231 cultures given 120 h acclimation time were not as resistant to doxorubicin as the 72 h acclimation time group, but they were still more resistant than their static hydrogel counterparts. Thus, we believe that there is an acute cellular response directing MDA-MB-231 cells into a highly resistant state between 24 – 72 h post-stiffening, but that this response is at least partially equilibrated by 120 h post-stiffening. MCF7 cultures had the highest observed resistance to doxorubicin when given only 24 h acclimation time post-stiffening, however only hydrogels stiffened from 2,000 Pa to 3,000 Pa for 72 h and 120 h acclimation periods were found to be statistically significantly different for the dynamically stiffened MCF7 cultures. Given that MCF7 cultures did not vary significantly within acclimation time groups, and only displayed small changes in resistance after the 24 h acclimation point, we believe this bolsters our findings from chapter 2 that the MCF7 cell line does not have a stiffness-dependent response to doxorubicin.

3.5 CONCLUSIONS

In this section, we have shown that the MDA-MB-231 cell line exhibits a stiffness-dependent resistance to doxorubicin and that drug resistance is affected by the duration of time that cells are exposed to an environment of a particular ECM stiffness. The MCF7 cell line, on the other hand, was not observed to share this stiffness-dependent resistance to doxorubicin, nor was it shown to be significantly affected by the duration of exposure to ECM stiffness. Taken together, this suggests that not all breast cancer cells will be significantly affected by stiffening of their ECM, but some subtypes may have their resistance to doxorubicin significantly increased by the tissue stiffening that occurs with breast cancer progression.

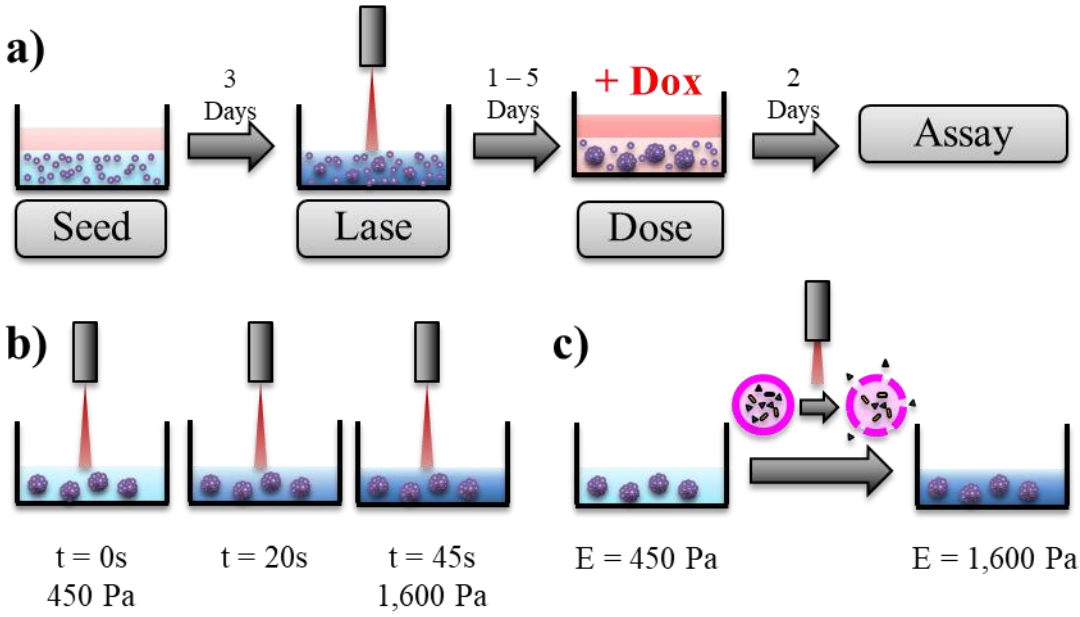

Fig. 3.1 – An alginate hydrogel platform was used to dynamically stiffen hydrogels to mimic progressive ECM stiffening. a) Cells were seeded into hydrogels and cultured for 3 days before dynamic stiffening with NIR light. After stiffening, cultures were given 1 – 5 days to acclimate to the new stiffness of the hydrogel before being exposed to doxorubicin for 2 days (48 h). Following treatment with doxorubicin, viability assays were performed to determine doxorubicin resistance. **b)** 450 Pa hydrogels were exposed to NIR light for 45 s to achieve ECM stiffness similar to 2,000 Pa static hydrogels. The same technique was used to stiffen 2,000 Pa hydrogels to 3,000 Pa. **c)** NIR light induces surface plasmon resonance in encapsulated gold nanorods (gold) to heat liposomes (pink) close to their gel-to-liquid transition temperature. This causes calcium (green) to leak from the liposomes and form additional alginate cross-links, thereby stiffening the hydrogel. The above figure was adapted from Joyce et al.[26].

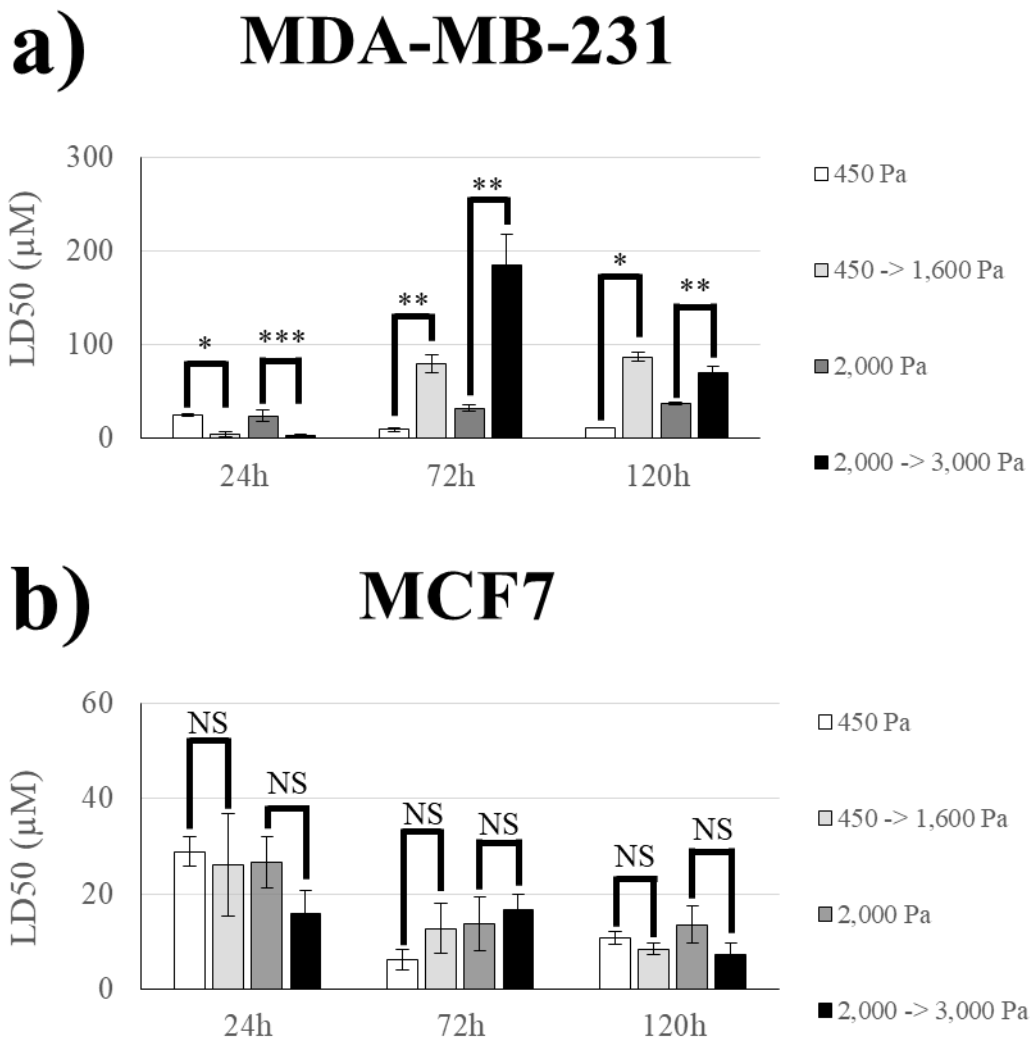

Fig. 3.2 – MDA-MB-231 cultures have an acclimation-dependent increase in resistance to doxorubicin. a) MDA-MB-231 and **b)** MCF7 cells were cultured in hydrogels with an initial stiffness of 450 Pa or 2,000 Pa for 3 days. On day 3, hydrogels either remained static (450 Pa or 2,000 Pa) or were stiffened (450 -> 1,600 Pa or 2,000 -> 3,000 Pa) using NIR light. Samples were then given 24 h to 120 h to acclimate to the hydrogel stiffness before 48 h treatment with doxorubicin. MDA-MB-231 cells showed a higher resistance to doxorubicin as hydrogel stiffness increased and this stiffness-dependent resistance was found to be partially dependent on duration of exposure to hydrogel stiffness. Comparable MCF7 samples did not show any significant change in resistance to doxorubicin across hydrogel stiffness or acclimation time. The above figure was adapted from Joyce et al.[26]. *p-value < 0.01; **p-value < 0.02; ***p-value < 0.03; NS = not significant, p-value >0.05

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Chapter 4: Molecular Characterization of Breast Cancer Cells Cultured in Soft and Stiff Hydrogels[4]

4.1 INTRODUCTION

Metastasis complicates disease treatment and decreases patient survivability, as the spread of tumors presents new locations that must be treated and these new locations will have different microenvironments that can influence resistance to treatment. Tumor metastasis occurs when cells from the primary tumor migrate to new locations and establish tertiary tumors. This process has been intensely studied in hopes of discovering methods to prevent or reduce the disease spreading through a patient's body. For breast cancer cells, this process has been well characterized (see[1-3] for more thorough reviews on the subject). Generally speaking, cells from a primary breast tumor will transition to a state where they are much more motile in a process called Epithelial-to-Mesenchymal transition (EMT). This transition is characterized by a loss of cell-cell adhesions, change from cuboidal to spindle shape, and increased expression of genes facilitating cell migration. For breast cancer, there have been six critical pathways identified that drive EMT: TGF-β (transforming growth factor- β), Wnt, Notch, Hippo, Hedgehog, and receptor tyrosine kinase pathways[2]. Our studies focused on three of these such pathways which are known to play a significant role in breast cancer initiation and metastasis.

TGF-β is a secreted protein that regulates key cellular processes including apoptosis, proliferation, and differentiation[4]. In very early stages of tumor development TGF-β signaling functions as a tumor suppressor, but as the disease progresses the TGF-β pathway has been shown to support tumor growth and metastasis through the induction of EMT[5]. Secreted TGF-β will bind to cell surface receptors and form complexes with Smad proteins which, when phosphorylated,

[4] Portions of this chapter were adapted from M. H. Joyce, C. Lu, E. R. James, R. Hegab, S. C. Allen, L. J. Suggs and A. Brock, "Phenotypic Basis for Matrix Stiffness-Dependent Chemoresistance of Breast Cancer Cells to Doxorubicin," *Front Oncol,* vol. 8, p. 337, 2018. A. B. and M. J. were responsible for the planning of the study and writing of the manuscript; C. L., E. J., M. J., R. H., and S. A.conducted the experiments; all authors were involved with the analysis of the manuscript.
Portions of this chapter include data that is currently in preparation for publication.

will translocate to the nucleus to drive transcription of target genes such as ZEB1/2, SNAI1/2, and TWIST1.

The Wnt pathway drives EMT through the downregulation of E-cadherin and up-regulation of Snail (SNAI1)[6], SLUG (SNAI2)[7], and TWIST[8] transcription factors. This can occur through the canonical β-catenin dependent pathway[9] or the non-canonical planar cell polarity and Wnt/Ca^{2+} pathways[10]. DiMeo et al. highlighted the necessity of Wnt signaling in EMT by demonstrating that over-expression of DKK1 (negative regulator of the Wnt pathway) was sufficient to decrease tumor formation in mice and reduce expression of EMT markers Slug and Twist[11]. A study of 158 invasive breast cancer samples showed that 99% of the samples examined had alterations in at least one antagonist Wnt pathway, further highlighting its clinical significance as a therapeutic target[12].

The Hippo pathway is a tumor suppressor pathway that contributes to tissue growth, differentiation, and regeneration. Yes-associated protein (YAP) and tafazzin (TAZ) are two oncogenes that make up part of the core transcriptional components, and both are highly regulated by the Hippo pathway. Phosphorylation of YAP/TAZ by LATS1/2 kinase suppresses their activity by causing them to accumulate in the cytoplasm of the cell where they undergo proteasome degradation[13]. Activation and subsequent nuclear translocation of YAP/TAZ due to inactivation of the Hippo pathway has been shown to lead to EMT[14-16] and increased proliferation[17-19]. Recent work has described how YAP/TAZ activation is mediated by the stiffness of their ECM through JNK[20-22], RHO[23,24], and integrin-mediated adhesion pathways[23,25,26]. Given cells in the mammary gland are already sensitive to ECM stiffness, they are an ideal platform to study YAP/TAZ induced EMT[27-29].

In this chapter, we sought to molecularly characterize the changes that occur when culturing mammary adenocarcinoma cells in soft (450 Pa) and stiff (2,000 Pa) ECM. Our studies focused on changes in markers of EMT due to recent findings that have found links between cells that have undergone EMT and increased chemoresistance in different tumor types.

4.2 Materials and Methods

4.2.1 Staining for EMT markers

Samples were fixed with a 15 min. exposure to 4% paraformaldehyde at room temperature. Blocking and permeabilization buffer (0.1% Triton X-100 and 1% bovine serum albumin [BSA] diluted in 1xPBS) was added to each sample and incubated at room temperature for 1 h. Following this, samples were incubated with primary antibody against YAP (Santa Cruz, sc-101199) or actin (Phalloidin488, Thermofisher, A12379) for 1 h at room temperature. Cell nuclei were stained with 300 nM DAPI (ThermoFisher, D1306) for 30 m at room temperature before being imaged on a Zeiss confocal or EVOS epifluorescent microscope.

4.2.2 Quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, 74104, Hilden, Germany) and was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) according to the manufacturer's instructions. Quantitative PCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems, A25743) with 20 ng cDNA input in 20 µl reaction volume run on a ViiA7 Real-Time PCR system (Applied Biosciences). B2M (beta-2-microglobulin) expression level was used for normalization as a housekeeping gene. The primers for beta-2-microglobulin (HK-B2M), Vimentin (VHPS-9912; Vimentin), and CDH1 (VHPS-1738; E-Cadherin) were designed and synthesized by RealTimePrimers.com (www.realtimeprimers.com). Delta CT (ΔCT) values for each sample were first normalized to the B2M house-keeping gene, and expression levels were analyzed pair-wise comparing samples cultured in 2,000 Pa hydrogels to the same cell type cultured in 450 Pa hydrogels.

4.2.3 Statistical Analysis and Mathematical Modeling

A two-tailed Student's t-test assuming unequal variance was used to compare samples, and a p-value of less than 0.05 was used to determine statistical significance. MatLab was used for plot generation and curve fitting.

4.3 RESULTS

4.3.1 Expression of EMT Markers

Quantitative real-time PCR was performed using a panel of well-known markers of EMT to evaluate how stiffness of the ECM would affect MDA-MB-231 and MCF7 cell state (**Fig. 4.1a**). We found that expression of E-Cadherin was decreased in both MDA-MB-231($p = 0.037$) and MCF7 ($p = 0.120$) cells cultured in 2,000 Pa hydrogels versus their 450 Pa counterparts (**Fig. 4.1b**), however only the MDA-MB-231 cells showed a statistically significant difference in expression with hydrogel stiffness. Decreased expression of E-Cadherin is a well-known marker of EMT, thus further supporting our findings that the stiffer hydrogels promote transition towards a mesenchymal phenotype. MCF7 cells cultured on 2,000 Pa hydrogels showed an increase in SNAIL1 ($p = 0.069$, **Fig. 4.1c**) and SLUG ($p = 0.006$, **Fig. 4.1d**) with a decrease in ZEB1 ($p = 0.445$, **Fig. 4.1e**) and vimentin ($p = 0.004$, **Fig. 4.1f**) expression compared to similar cultures grown in 450 Pa hydrogels. MDA-MB-231 samples cultured in 2,000 Pa hydrogels showed a decrease in all SNAIL1 ($p = 0.001$, **Fig. 4.1c**), SLUG ($p = 0.017$, **Fig. 4.1d**), ZEB1 ($p = 0.002$, **Fig. 4.1e**), and vimentin ($p = 0.018$, **Fig. 4.1f**) additional markers tested when compared to their 450 Pa counterparts.

We took the same data and normalized all samples to the epithelial model (MCF7) cultured on the softest stiffness (450 Pa) (**Fig. 4.2a**). What we found was that there were significant differences in expression between our mesenchymal (MDA-MB-231) and epithelial (MCF7) cell models for all genes tested. Expression of E-Cadherin was much higher in MCF7 cultures and culturing the highly epithelial MCF7 cells in 2,000 Pa hydrogels was not sufficient to decrease

their expression of E-Cadherin to similar levels seen in MDA-MB-231 cells cultured in either 450 Pa ($p = 0.002$) or 2,000 Pa ($p = 0.002$) hydrogels (**Fig. 4.2b**). SNAIL1 expression was also found to be higher in MCF7 cultures compared to MDA-MB-231 ($p = 0.002$ for 450 Pa and $p = 0.0002$ for 2,000 Pa hydrogels, **Fig. 4.2c**). SLUG ($p = 0.0003$ for 450 Pa and $p = 0.002$ for 2,000 Pa hydrogels, **Fig. 4.2d**), ZEB1 ($p = 0.0004$ for 450 Pa and $p = 0.0001$ for 2,000 Pa hydrogels, **Fig. 4.2e**), and Vimentin ($p = 0.0005$ for 450 Pa and $p = 0.0001$ for 2,000 Pa hydrogels, **Fig. 4.2f**) all had higher overall expression in MDA-MB-231 cultures and were found to decrease with expression ($p = 0.017$ for SLUG, $p = 0.002$ for ZEB1, $p = 0.018$ for vimentin) as stiffness increased. SLUG was shown to increase with stiffness ($p = 0.006$, **Fig. 4.2d**) for MCF7 cultures, but expression of ZEB1 (**Fig. 4.2e**) and vimentin (**Fig. 4.2f**) did not show a statistically significant difference between 450 Pa and 2,000 Pa cultures.

4.3.2 Increasing hydrogel stiffness leads to nuclear localization of YAP

Samples grown on 450 or 2,000 Pa hydrogels were stained with YAP antibodies as a marker of mesenchymal phenotype. High levels of YAP nuclear localization confirmed that 231 cultures (**Fig. 4.3a**) exhibited higher markers of mesenchymal phenotype than their MCF7 (**Fig. 4.3b**) counterparts. The mean fluorescent intensity (MFI) of YAP-labeled nuclei was MFI = 930 a.u. in 231 cells cultured in 450 Pa hydrogels and MFI = 2,030 a.u. for the same cells cultured in 2000 Pa hydrogels. Given the 231 cells grown on 2,000 Pa hydrogels showed higher levels of YAP nuclear localization than similar cultures on 450 Pa hydrogels ($p = 1.36E-22$), we believe that a stiffer ECM promotes the mesenchymal phenotype for these cells. Similar findings were observed in MCF7 cultures, but to a much lesser extent (MFI = 22 a.u. for cells cultured in 450 Pa hydrogels and MFI = 28 a.u. for cells cultured in 2,000 Pa hydrogels, $p = 0.0001$).

4.3.3 Mathematical models suggests stiffness-induced resistance to doxorubicin is not fully explained by changes in proliferation rates

MDA-MB-231 cells expressing nuclear localized red fluorescent protein were cultured in 450 Pa, 900 Pa, 1,400 Pa, or 2,000 Pa hydrogels ($n = 11$ for all groups) seeded in a 96-well plate

and imaged every 4 h to track cell growth. Cells were cultured for 6 days to collect growth dynamics data (**Fig. 4.4**). All cultures were then exposed to 10 μM doxorubicin for 48 h, before the drug was replaced with standard growth medium and monitored for an additional 5 days (11 d, 262 h total, **Fig. 4.5**). Growth trajectories (g_{eff}) were calculated by fitting cell count data from time points 48 h – 152 h to a single exponential growth model (**Fig. 4.6a,b**). Post-treatment growth trajectories (k_{eff}) were calculated similarly, by fitting cell count data from time points 152 h – 262 h to a single exponential death model (**Fig. 4.6a,b**). Our results from this show that the growth rate of MDA-MB-231 cells is inversely correlated with hydrogel stiffness (**Fig. 4.6c**). Using the same data, we modeled how sensitivity to doxorubicin would be affected by a two-population model that accounted for cells expressing both the epithelial and mesenchymal phenotype with the option to transition between the two states (**Fig. 4.7a**). Here we set the mesenchymal growth rate (g_m = 0.0013 cells per hour) and the transition from mesenchymal-to-epithelial rate (k_{me} = 0.0013) to allow us to fit for the epithelial growth rate (g_e = 0.0335) and transition rate from epithelial-to-mesenchymal phenotype (k_{em}) (**Fig. 4.7b**). Our model showed that the transition rate from epithelial-to-mesenchymal phenotype (k_{em}) increased with hydrogel stiffness (**Fig. 4.7c**). We were then able to calculate drug sensitivity (**Fig. 4.8a**) by fitting the equations shown in **Fig. 4.8b** to find the average death rate (d_E and d_M) of each phenotype following treatment with doxorubicin. Our results show that cells expressing the epithelial phenotype are more sensitive to doxorubicin than cells expressing a mesenchymal phenotype (**Fig. 4.8c**).

4.4 DISCUSSION

Previous studies have shown links between EMT and increased resistance to certain chemotherapeutics. We sought to see if this was true for breast cancer cells treated with doxorubicin, a drug with clinical relevance to this disease known to induce chemotherapeutic resistance. To this end, we cultured breast cancer cells known to exhibit the epithelial (MCF7) and mesenchymal (MDA-MB-231) phenotypes in hydrogels that mimicked the stiffness of ECM ranging from healthy mammary tissue (450 Pa) to early stage breast tumor (2,000 Pa). Using

quantitative real-time PCR, we found that SNAIL1 and SLUG increase with ECM stiffness in MCF7 cells, but ZEB1 and vimentin decrease with ECM stiffness. This, paired with the fact that expression of all markers examined decreased for MDA-MB-231 cells, suggests that matrix stiffness alone may not be sufficient to fully induce EMT in certain breast cancer cells. Both cell lines showed loss of cell-cell adhesions, and MCF7 cells upregulated expression of additional negative regulators of E-cadherin (SNAIL1 and SLUG). However, neither MDA-MB-231 nor MCF7 cells showed increased expression of ZEB1 or vimentin. ZEB1 has recently been shown to induce chemoresistance in breast cancer cells through activation of ataxia-telangiectasia mutated (ATM) kinase[30], however our results suggest that this is not the mechanism governing the stiffness-dependent resistance seen in the MDA-MB-231 samples.

A recent study looking at partial EMT in pancreatic ductal adenocarcinoma (PDAC), breast cancer, and colorectal cancer cell lines showed that cells classified as having undergone partial EMT did not always show a significant decrease in expression of E-cadherin, rather they re-localized it from the membrane to the interior of the cell[31]. Knowing this, we turned our attention to another marker of EMT – nuclear localization of YAP. YAP nuclear localization and subsequent loss of E-cadherin are classical markers of cells undergoing EMT, so we cultured MCF7 and MDA-MB-231 cells in 450 Pa and 2,000 Pa to examine how ECM stiffness would affect YAP nuclear localization. We found that both cell lines showed higher nuclear localization of YAP when cultured in stiffer hydrogels. Similar findings were found by Rice et al.[32] in pancreatic ductal adenocarcinoma cells grown in hydrogels of increasing stiffness. Thus, this further supports our hypothesis that ECM stiffness increases breast cancer cell resistance to doxorubicin through partial induction of EMT.

4.5 Conclusion

We found that quantitative analysis of EMT-associated gene expression suggests that ECM stiffness alone is not sufficient to induce MCF7 cells to fully undergo EMT or to prompt further EMT in MDA-MB-231 cells. Our results find that the decreased expression of ZEB1 in MDA-

MB-231 cells suggests that the stiffness-dependent resistance these cells displayed in chapter 2 and chapter 3 is likely not due to ZEB1 activation of ATM. Mathematical modeling of MDA-MB-231 response to doxorubicin points towards a link between EMT rate and ECM stiffness as the driver of the observed stiffness-dependent resistance.

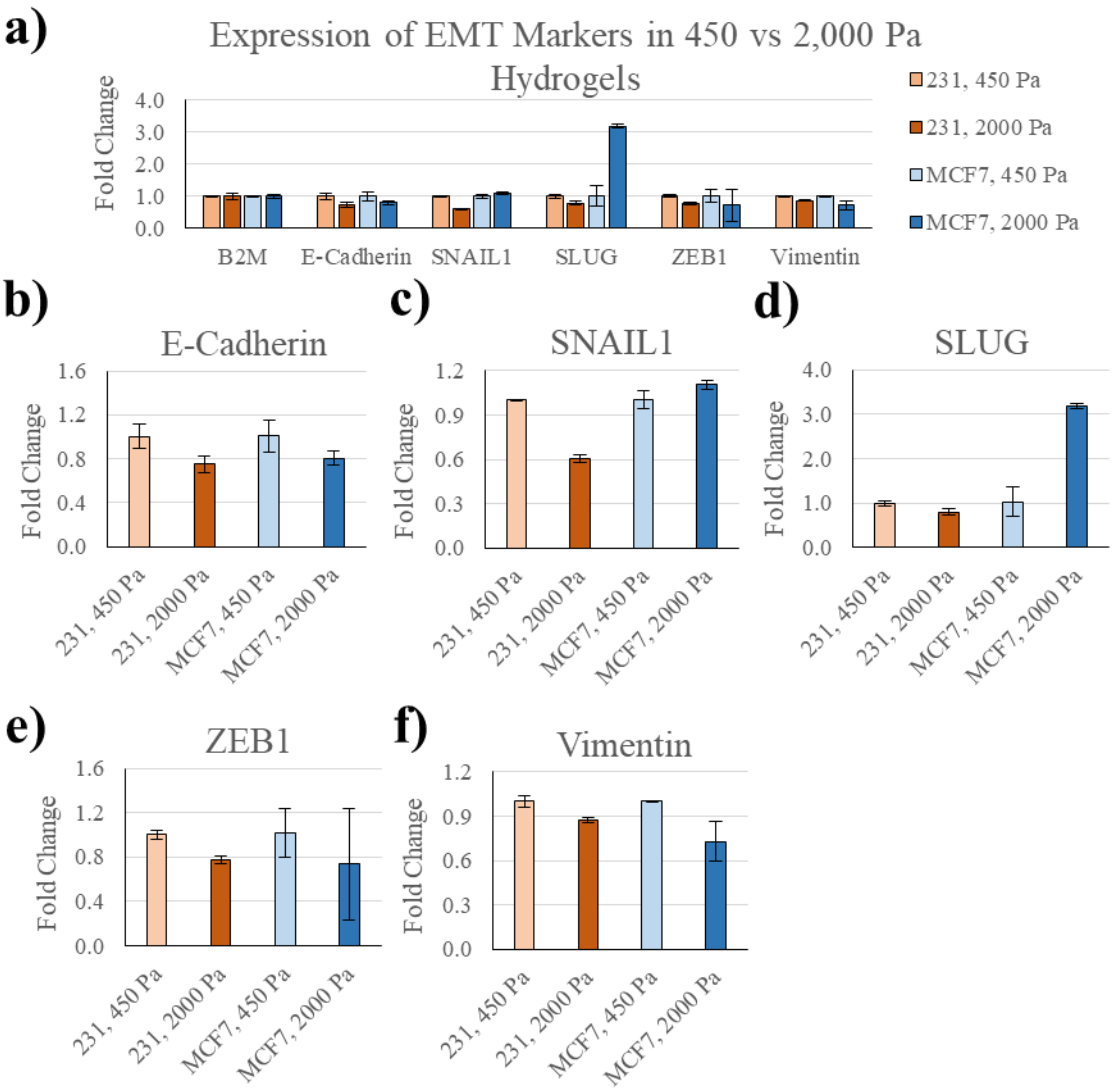


Fig. 4.1 – Expression of EMT markers suggests ECM stiffness promotes hybrid cell state in MDA-MB-231 and MCF7 cells. **a)** The expression of five EMT-related genes was examined in MDA-MB-231 (231)/MCF7 cells cultured in 450 Pa vs 2,000 Pa hydrogels using quantitative PCR (qPCR). Cells were cultured in hydrogels for 6 days before RNA was isolated from samples and prepared for qPCR. All primers were normalized to the beta-2-microglobulin (B2M) house-keeping gene. **b)** E-cadherin expression was shown to decrease when both cell lines were cultured in 2,000 Pa hydrogels vs 450 Pa hydrogels, though only the MDA-MB-231 ($p = 0.037$) cultures showed a significant decrease whereas the MCF7 ($p = 0.120$) cultures did not. **c)** SNAIL1 expression was shown to significantly decrease for MDA-MB-231 samples ($p = 0.001$), and while expression increased in MCF7 cultures ($p = 0.069$) it was not found to be statistically significant. **d)** Expression of SLUG (SNAI2) was shown to significantly decrease for MDA-MB-231 cultures ($p = 0.017$), but increase 3-fold for MCF7 cultures ($p = 0.006$). **e)** ZEB1 was shown to have less expression in MDA-MB-231 cells ($p = 0.002$), but were not shown to be statistically different for MCF7 cells ($p = 0.445$) cultured in stiffer hydrogels. **f)** Expression of vimentin was found to be significantly decreased for both MDA-MB-231 ($p = 0.018$) and MCF7 ($p = 0.004$) cells cultured in 2,000 Pa hydrogels compared to their counterparts cultured in 450 Pa hydrogels.

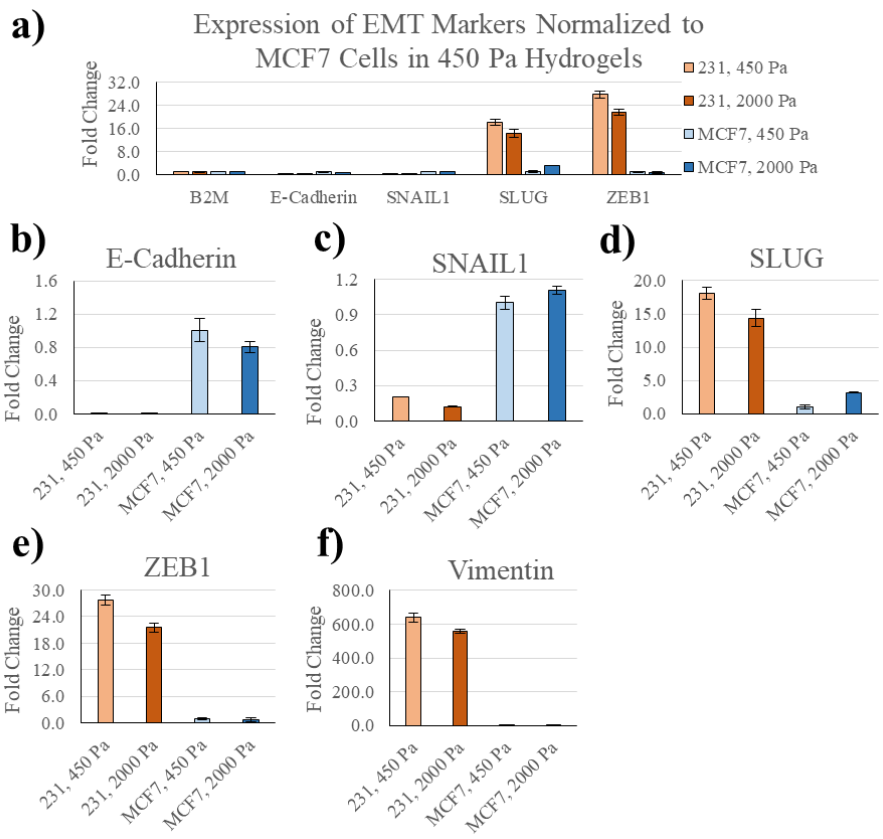


Fig. 4.2 – Expression of EMT markers compared to epithelial cells on soft hydrogels. **a)** The expression of five EMT-related genes was examined in MDA-MB-231 (231)/MCF7 cells cultured in 450 Pa vs 2,000 Pa hydrogels using quantitative PCR (qPCR). All primers were normalized to the beta-2-microglobulin (B2M) house-keeping gene and compared against the MCF7 cells cultured in 450 Pa hydrogels. **b)** E-cadherin expression is much higher in MCF7 cells for both 450 Pa ($p = 0.007$) and 2,000 Pa ($p = 0.002$) hydrogels. **c)** SNAIL1 expression overall is much lower in MDA-MB-231 cultures ($p = 0.002$ for 450 Pa and $p = 0.0002$ for 2,000 Pa hydrogels). SNAIL1 further decreases in MDA-MB-231 cultures ($p = 0.001$) as stiffness increases but increases with stiffness in MCF7 cultures ($p = 0.069$). **d)** Expression of SLUG (SNAI2) is much higher in MDA-MB-231 cultures ($p = 0.0003$ for 450 Pa and $p = 0.002$ for 2,000 Pa hydrogels). Again, SLUG further decreases in MDA-MB-231 cultures ($p = 0.017$) as stiffness increases but increases with stiffness in MCF7 cultures ($p = 0.006$). **e)** ZEB1 expression is much higher in MDA-MB-231 cultures overall ($p = 0.0004$ for 450 Pa and $p = 0.0001$ for 2,000 Pa hydrogels). Here we observe a stiffness-dependent decrease in ZEB1 for both MDA-MB-231 ($p = 0.002$) and MCF7 ($p = 0.445$), though differences in MCF7 cultures were not found to be statistically significant. **f)** Vimentin is dramatically more expressed in MDA-MB-231 cultures ($p = 0.0005$ for 450 Pa and $p = 0.0001$ for 2,000 Pa hydrogels). A stiffness-dependent decrease in vimentin was observed in both MDA-MB-231 ($p = 0.018$) and MCF7 ($p = 0.076$) cultures, though the difference in MCF7 cultures was not significant.

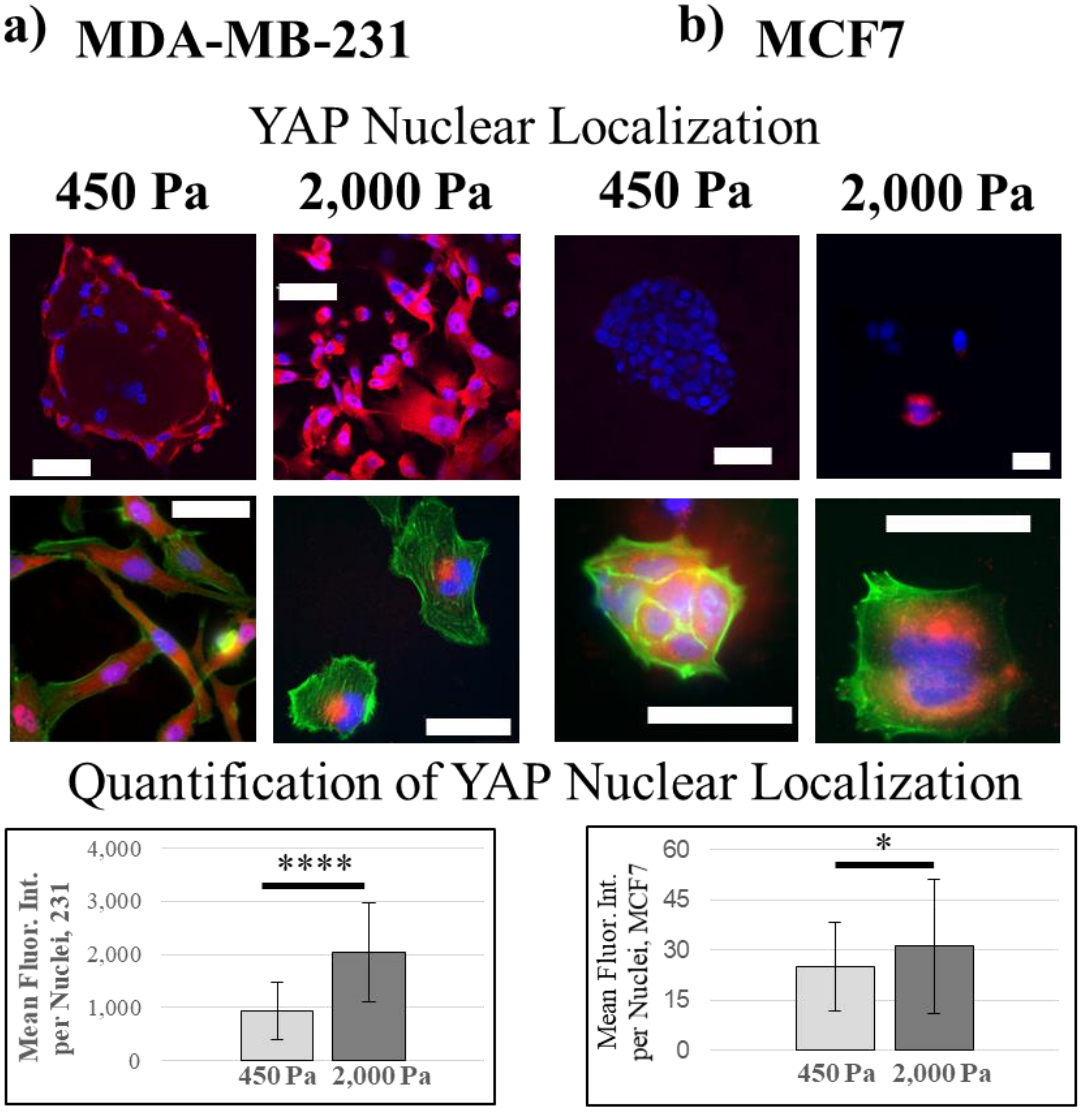


Fig. 4.3 – Stiffer ECM increases nuclear localization of YAP and decreases expression of E-Cadherin. **a)** MDA-MB-231 and **b)** MCF7 cells were cultured on 450 Pa or 2,000 Pa hydrogels for 3 days before fixation and staining with YAP (red) and actin (phalloidin-488, green) antibodies, as well as DAPI (blue). Images were captured using confocal microscopy and analysis was done in ImageJ to determine nuclear localization of YAP (n = 123 for MDA-MB-231 cells cultured in 450 Pa hydrogels, n = 119 for MDA-MB-231 cells cultured in 2,000 Pa hydrogels, n = 72 for MCF7 cells cultured in 450 Pa hydrogels, n = 90 for MCF7 cells cultured in 2,000 Pa hydrogels). There is a stiffness-dependent increase in nuclear localization of YAP for both MDA-MB-231 ($p = 1.38E-22$) and MCF7 ($p = 0.02$) cultures, though the increase in MCF7 cultures is small. MDA-MB-231 cultures showed significantly higher expression of nuclear YAP compared to similar MCF7 cultures ($p = 2.37E-37$ for 450 Pa and $p = 4.44E-46$ for 2,000 Pa). Scale bar = 50 µm. *p-value < 0.05; ****p-value << 0.001

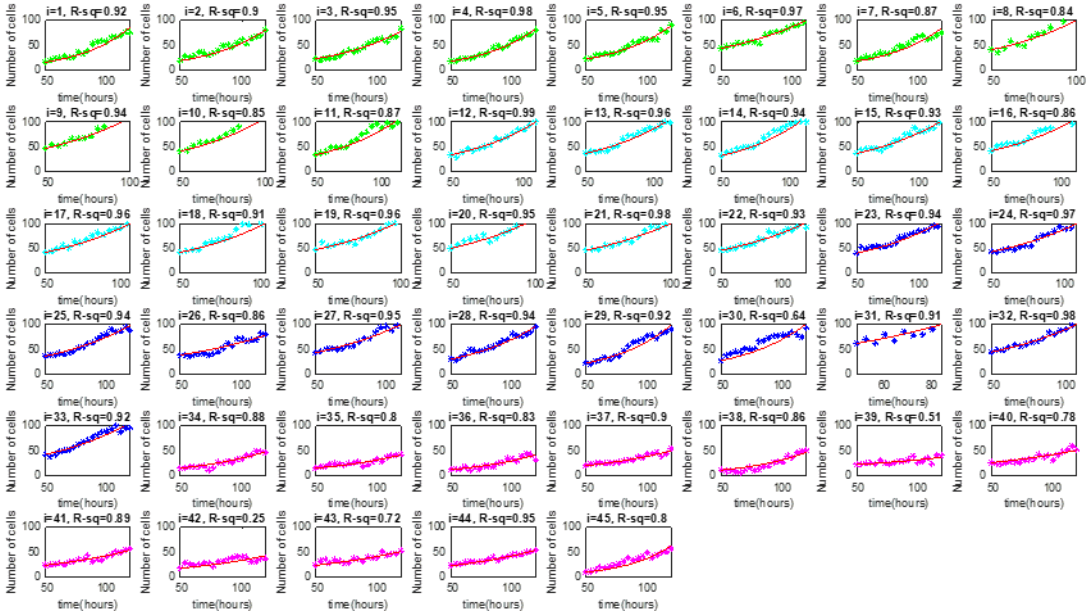


Fig. 4.4 – Growth dynamics of MDA-MB-231 cells in hydrogels of varying stiffness before treatment with doxorubicin. MDA-MB-231 cells were seeded into 450 (green), 900 (cyan), 1400 (blue), or 2000 (pink) Pa hydrogels and cultured for 6 days in an Incucyte S3 incubator. Images were collected every 4 h and cells were counted using the S3 on-board software.

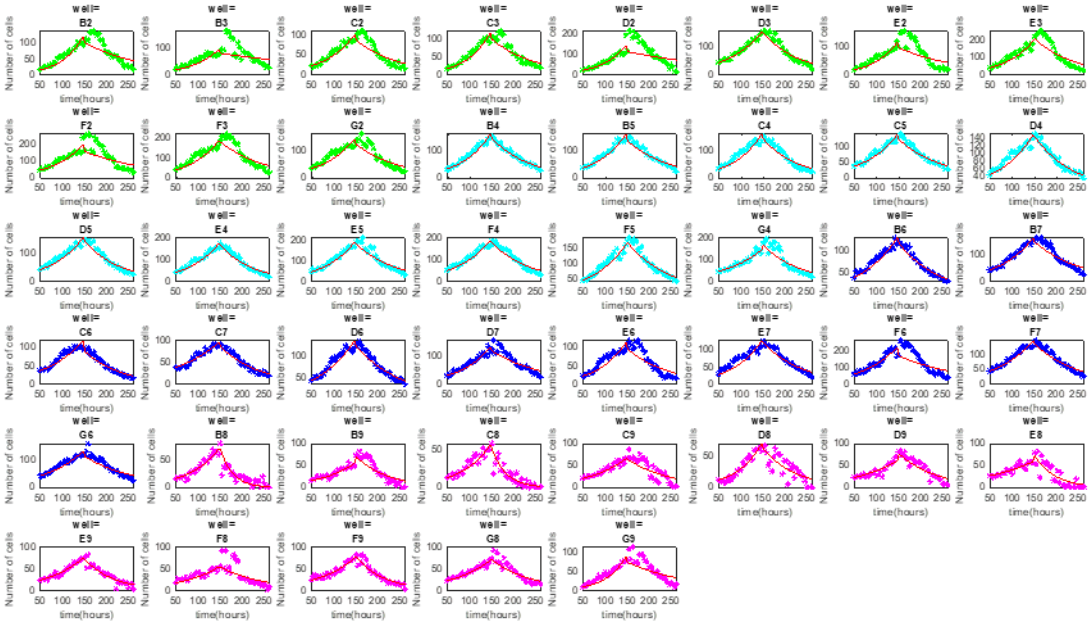


Fig. 4.5 – Growth dynamics of MDA-MB-231 cells in hydrogels of varying stiffness following treatment with doxorubicin. Following 6 days of standard growth in 450 (green), 900 (cyan), 1400 (blue), or 2000 (pink) Pa hydrogels, cells were exposed to 10 μM doxorubicin for 48 h. Cultures were monitored for an additional 5 days to collect growth dynamics information following treatment with doxorubicin. Images were collected every 4 h in an Incucyte S3 incubator and cells were counted using the S3 on-board software.

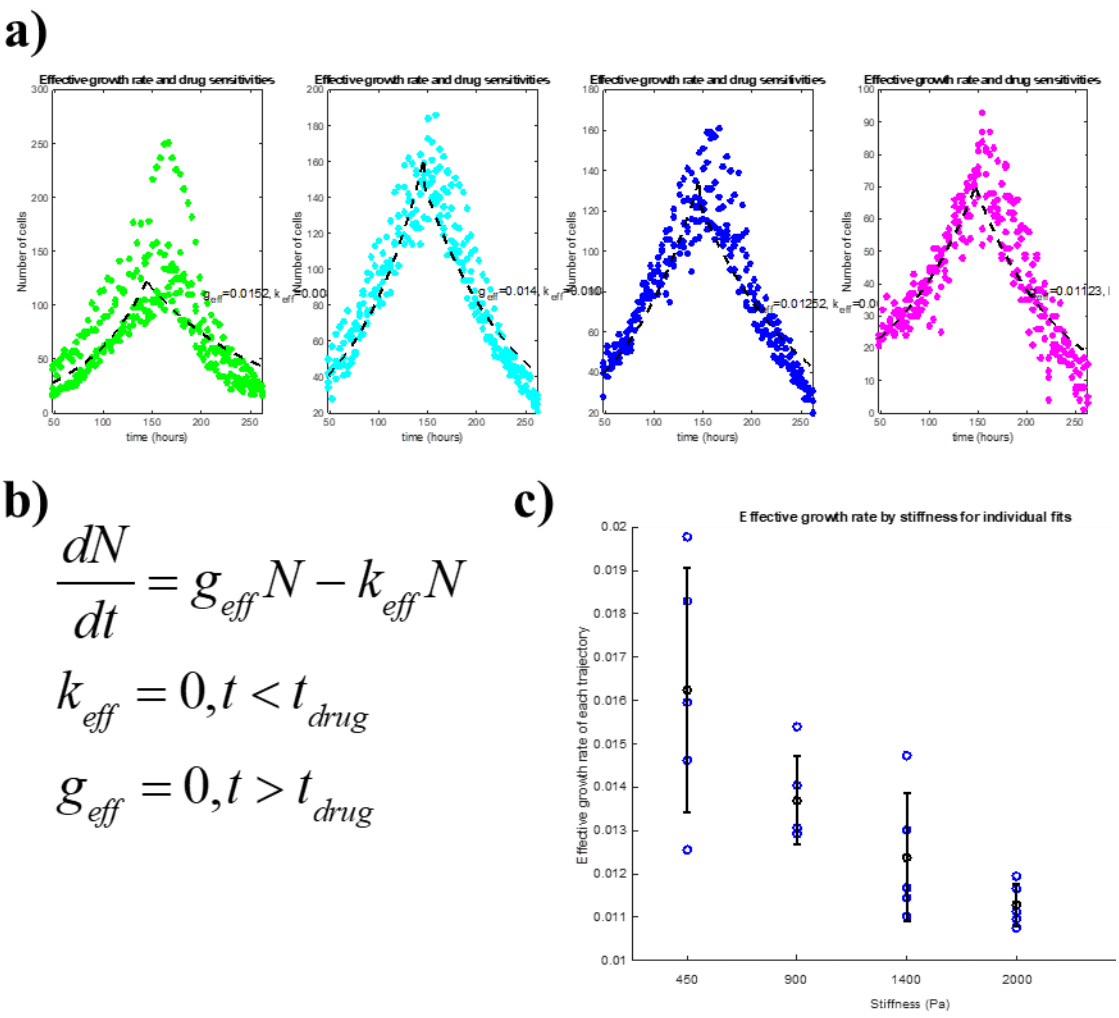


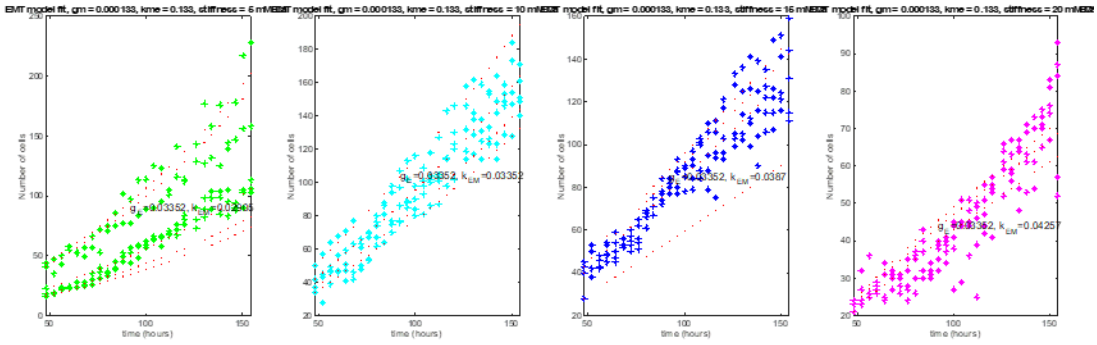
Fig. 4.6 – Proliferation rate of MDA-MB-231 cells decreases with increased hydrogel stiffness. **a)** Data collected between 48 h and 152 h time points was fit to **b)** a single exponential growth model to calculate the effective growth rate for cultures pre-treatment. The cell count data from 152 h to 262 h time points, was fit to a single exponential death model to calculate the effective growth rate of cells following 48 h exposure to doxorubicin. c) Our results show that growth rate of MDA-MB-231 cells decreases as the stiffness of the hydrogel increases. The error bars shown represent the 95% confidence interval, n = 5 for each hydrogel stiffness.

a)

$$\frac{dE}{dt} = g_E E - k_{EM}(\sigma)E + k_{ME} M$$

$$\frac{dM}{dt} = g_M M + k_{EM}(\sigma)E - k_{ME} M$$

b)



c)

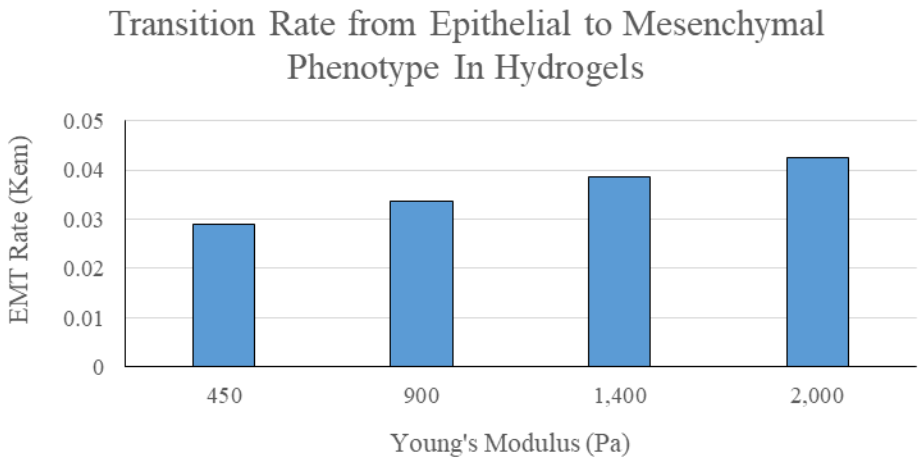
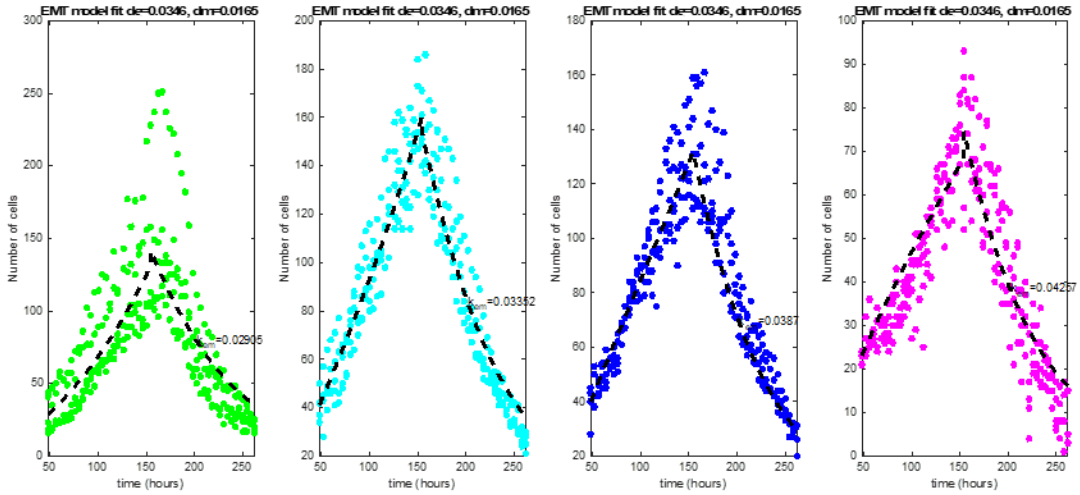


Fig. 4.7 – Fitting data to a two-population EMT model shows rate of EMT increases with hydrogel stiffness. **a)** Growth data collected from the Incucyte S3 was fit to a two-population model that assumed populations of epithelial (E) and mesenchymal (M) were present and that they could transition from one E to M (k_{EM}) or vice-versa (k_{ME}). **b)** Assuming a mesenchymal growth rate (g_M) of 0.0013 cells per hour and k_{ME} of 0.0013, we fit the data to calculate epithelial growth rate (g_E) and k_{EM}. **c)** Our mathematical model shows that the rate of transition from E to M (k_{EM} or EMT) increases with hydrogel stiffness.

a)



b)

$$\frac{dE}{dt} = g_E E - k_{EM}(\sigma)E + k_{ME}M - d_E E$$

$$\frac{dM}{dt} = g_M M + k_{EM}(\sigma)E - k_{ME}M - d_M M$$

c)

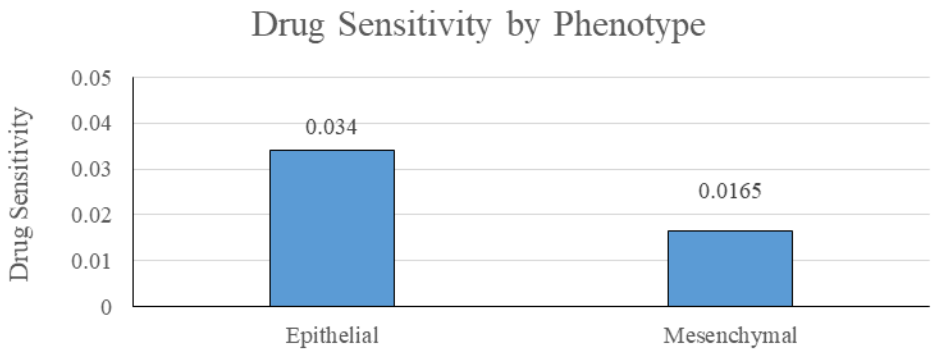


Fig. 4.8 – Cells with mesenchymal phenotype have a lower death rate in response to treatment with doxorubicin. **a)** We fit our data to the equations in **b)** using previously calculated variables to determine the sensitivity of epithelial (E) and mesenchymal (M) populations to doxorubicin. **c)** Our model shows that mesenchymal cells are more resistant to doxorubicin. This suggests that cells undergoing transitioning from E to M (k_{EM} or EMT) would have a survival advantage and increase the overall resistance of the cell entire population.

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Appendix

Cell Type	Stiffness (Pa)	LD50 (μM)	Std Dev. (μM)
231	450	9.59	1.87
	450 → 1,600	79.99	9.54
	2,000	32.24	3.45
	2,000 → 3,000	184.97	32.28
	Monolayer	26.57	2.91
MCF7	450	6.02	2.19
	450 → 1,600	12.68	5.18
	2,000	12.07	6.88
	2,000 → 3,000	16.60	3.26
	Monolayer	4.44	2.00

Cell Type	Stiffness (Pa)	LD50 (μM)	Std Dev. (μM)
231	450 → Monolayer	14.23	0.65
	2,000 → Monolayer	11.24	2.34
MCF7	450 → Monolayer	1.55	0.61
	2,000 → Monolayer	3.33	2.44

Table A1 – Doxorubicin sensitivity for MCF7 and 231 across culture conditions. Dose response curves of 231 and MCF7 cells cultured in static hydrogels, dynamic hydrogels, and monolayer culture conditions following 48h exposure to Doxorubicin. Live and Dead cells were counted using a Nexcelom Cellometer with AOPI stain.

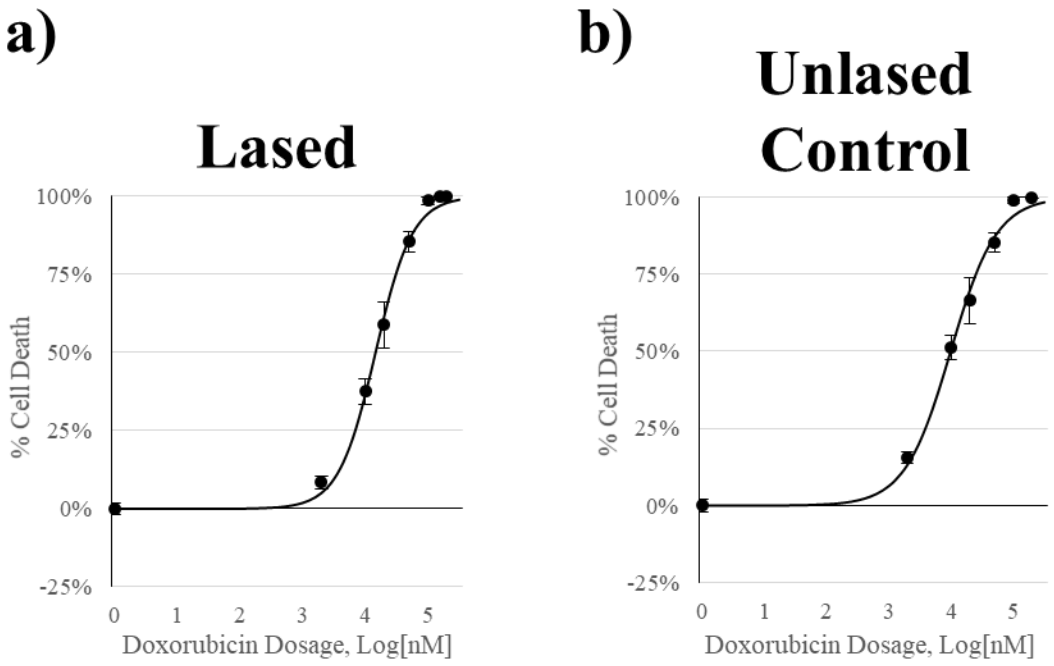

Fig. A1 – Exposure to Near Infrared (NIR) light does not significantly affect response to doxorubicin. MDA-MB-231 cells were cultured as a monolayer (2D) on tissue culture plastic before being exposed to NIR light for 45 s. Samples were cultured an additional 2 days after lasing prior to treatment with doxorubicin. Staining with acridine orange/propidium iodide (AOPI) was used to quantify the ratio of live/dead cells. **a)** Cells that were exposed to NIR light did not show a significant difference ($p = 0.064$) in resistance to doxorubicin compared to **b)** control samples that were not exposed to NIR light. $n = 3$

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CHAPTER 1

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CHAPTER 2

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